(12)   EUROPEAN PATENT APPLICATION

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(54) Improvements in or relating to antibiotic-producing microorganisms.

(57) A means for increasing the antibiotic-producing ability of an antibiotic-producing microbial host cell is disclosed. The method involves transforming an antibiotic-producing microorganism with a recombinant DNA cloning vector that codes for the expression of a rate-limiting antibiotic biosynthetic enzyme or other gene product.

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Improvements In or Relating To
Antibiotic-Producing Microorganisms

The present invention provides a novel method
5   for increasing the antibiotic-producing ability of an
antibiotic-producing organism. The method involves
transforming a microbial host cell with a DNA sequence
that codes for the expression of a gene product that is
rate-limiting in the desired antibiotic's biosynthetic
10   pathway. The invention also provides related DNA
sequences that code for antibiotic biosynthetic gene
products, recombinant DNA expression vectors, and
transformed microbial host cells.

The present invention represents an early and
15   significant commercial exploitation of recombinant DNA
technology in antibiotic-producing organisms such as
streptomycetes. Prior to the present invention, the
development and exploitation of recombinant DNA technol-
ogy has been limited, for the most part, to the expres-
20   sion of specific polypeptides in E. coli and, in some
instances, mammalian cells. These advances led to the
comparatively simple expression of heterologous gene
products such as human insulin A and B chains, human
proinsulin, human growth hormone, human protein C, human
25   tissue plasminogen activator, bovine growth hormone, and
several other compounds of potential value. In each
case, heterologous gene expression is more or less
independent and does not interact with, take part in, or
modulate operative biosynthetic pathways. Recombinant
30   DNA technology can now be applied to improve selected

biosynthetic pathways for the expression of increased yields of antibiotics or antimicrobial precursors.

Most recombinant DNA technology applied to streptomycetes and other antibiotic-producing organisms

5   has been limited to the development of cloning vectors. Early attempts include the disclosures of Reusser U.S. Patent No. 4,332,898 and Manis et al. U.S. Patent Nos. 4,273,875; 4,332,900; 4,338,400; and 4,340,674. Transformation of streptomycetes was not disclosed or taught

10 in these early references. Improved vectors showing greater potential for use in antibiotic-producing organisms were disclosed, for example, by Fayerman et al. in U.S. Patent No. 4,513,086; and Nakatsukasa et al. in U.S. Patent Nos. 4,513,085 and 4,416,994. These

15 improved vectors contain markers that are selectable in streptomycetes, can be used to transform many important Streptomyces strains, and constitute the tools required for conducting more complicated gene cloning experiments.

20   One such experiment was recently reported by Hopwood et al., 1985, in Nature 314:642. Although Hopwood et al. reported the production of novel hybrid antibiotic pigments, the disclosure does not focus on increasing the antibiotic-producing ability or biosyn-

25 thetic efficiency of a given host cell but instead describes the transferring of actinorhodin pigment biosynthetic genes from one Streptomyces strain to another.

The present invention is particularly useful

30 because it allows commercial application of recombinant

DNA technology to streptomycetes and other antibiotic-
producing organisms.   Because over half of the clinically
important antibiotics are produced by streptomycetes, it
is especially desirable to develop methods that are

5   applicable to these organisms. The present invention
provides such methods and allows for the cloning of
genes both for increasing the antibiotic-producing
ability as well as for the production of new antibiotics
and antibiotic precursors in an antibiotic-producing

10   organism.
         For purposes of the present invention, the
following terms are as defined:

Antibiotic - a substance produced by a microorganism

15   that, either naturally or with limited chemical modifi-
cation, inhibits or prevents the growth of or kills
another microorganism or eukaryotic cell.

Antibiotic Biosynthetic Gene - a DNA segment that

20   encodes an enzymatic activity or encodes a product that
regulates expression of an enzymatic activity and which
is necessary for an enzymatic reaction for converting
primary metabolites to antibiotic intermediates, which
also may possess antibiotic activity, and perhaps then

25   to antibiotics.

Antibiotic Biosynthetic Pathway - the entire set of
antibiotic biosynthetic genes and biochemical reactions
necessary for the process of converting primary metabo-

lites to antibiotic intermediates and then to
antibiotics.

Antibiotic-Producing Microorganism - any organism,
5   including, but not limited to <u>Actinoplanes</u>,
<u>Actinomadura</u>, <u>Bacillus</u>, <u>Cephalosporium</u>, <u>Micromonospora</u>,
<u>Penicillium</u>, <u>Nocardia</u>, and <u>Streptomyces</u>, that either
produces an antibiotic or contains genes that, if
expressed, would produce an antibiotic.
10

Antibiotic Resistance-Conferring Gene - a DNA segment
that encodes an activity that confers resistance to an
antibiotic.

15 ApR - the ampicillin-resistant phenotype or gene confer-
ring same.

Host Cell - an organism, including a viable protoplast
thereof, which can be transformed with a recombinant DNA
20 cloning vector.

NmR - the neomycin-resistant phenotype or gene confer-
ring same.

25 Operation of Antibiotic Biosynthetic Pathway - the
expression of antibiotic biosynthetic genes and the
related biochemical reactions required for the conver-
sion of primary metabolites into antibiotics.

Recombinant DNA Cloning Vector - any selectable and autonomously replicating or chromosomally integrating agent, including but not limited to plasmids and phages, comprising a DNA molecule to which additional DNA can be
5   or has been added.

rep - as used in the Figures, a <u>Streptomyces</u> plasmid origin of replication.

10   Restriction Fragment - any linear DNA generated by the action of one or more restriction enzymes.

Sensitive Host Cell - a host cell, including a viable protoplast thereof, which cannot grow in the presence of
15   a given antibiotic without a DNA segment that confers resistance thereto.

Transformant - a recipient host cell, including a viable protoplast thereof, which has undergone transformation.
20

Transformation - the introduction of DNA into a recipient host cell, including a viable protoplast thereof, that changes the genotype of the recipient cell.

25   tsr - the thiostrepton-resistant phenotype or gene conferring same.

The plasmid and chromosomal maps depicted in the Figures are drawn approximately to scale. However,
30   the tylosin biosynthetic genes, although linked, are

scattered across a large segment of DNA. Therefore,
detailed restriction site mapping data exists only for
small regions of the large tylosin biosynthetic
gene-containing DNA fragment. The maps do not necessar-
5   ily provide an exhaustive listing of all the cut sites
of a given restriction enzyme. The location of individ-
ual genes, represented by line segments on the maps, was
determined by deletion mapping and thus only approxi-
mates the exact location of a given gene.
10   Figure 1 - The Tylosin Biosynthetic Pathway.
                Figure 2 - Restriction Site and Function Map
of Plasmid pHJL280.
                Figure 3 - Restriction Site and Function Map
of Plasmid pHJL284.
15   Figure 4 - Restriction Site and Function Map
of Plasmid pHJL309.
                Figure 5 - Restriction Site and Function Map
of Plasmid pHJL311.
                Figure 6 - Restriction Site and Function Map
20   of Plasmid pHJL315.
                Figure 7 - Chromosomal Organization of the
Tylosin Biosynthetic Genes.

The present invention provides a method for increasing
25   the antibiotic- or antibiotic precursor-producing
ability of an antibiotic-producing microorganism, which
comprises culturing a microorganism which produces an
antibiotic or antibiotic precursor by a biosynthetic
pathway, said microorganism being transformed with a DNA
30   cloning vector or portion thereof which contains an

antibiotic or antibiotic-precursor biosynthetic gene
coding for expression of a rate-limiting enzyme or gene
product of the biosynthetic pathway, under conditions
suitable for cell growth, expression of the antibiotic

5   or antibiotic-precursor biosynthetic gene and production
of the antibiotic or antibiotic precursor, provided that
the culturing process provides an increase in the
antibiotic-producing ability of the microorganism.

10 The invention further provides related antibiotic
biosynthetic genes, recombinant DNA cloning vectors, and
antibiotic or antibiotic precursor-producing microorgan-
isms transformed with the genes and vectors.

15 Further, there is provided a process for preparing an
antibiotic, an antibiotic precursor, or a pharmaceuti-
cally acceptable salt thereof, which comprises culturing
a microorganism which produces an antibiotic or antibi-
otic precursor through an antibiotic biosynthetic

20 pathway, said microorganism being transformed with a DNA
cloning vector, or portion thereof, in a culture medium
containing assimilable sources of carbon, nitrogen and
inorganic salts under aerobic fermentation conditions
characterized in that the DNA cloning vector, or portion

25 thereof, comprises an antibiotic biosynthetic gene which
codes for the expression of a rate-limiting enzyme or
gene product of the antibiotic biosynthetic pathway,
said antibiotic biosynthetic gene being expressed under
fermentation conditions providing for an increase in the

30 antibiotic-producing ability of the microorganism.

The methods of the present invention are widely applicable to all antibiotic-producing organisms. The following tables provide a non-exhaustive list of antibiotic producing organisms to which the present invention may

5   apply.

TABLE I

Aminocyclitol Antibiotic-Producing Organisms

	Organism	Antibiotic
5		
	Bacillus	
	various species	various aminocyclitols
10	**Micromonospora**	
	various species	gentamycins
	Saccharopolyspora	
	various species	various aminocyclitols
15		
	Streptomyces	
	albogriseolus	neomycins
	albus var. metamycinus	metamycin
	aquacanus	N-methyl hygromycin B
20	atrofaciens	hygromycins
	bikiniensis	streptomycin
	bluensis var. bluensis	bluensomycin
	canus	ribosyl paromamine
	catenulae	catenulin
25	chrestomyceticus	aminosidine
	crystallinus	hygromycin A
	erythrochromogenes	
	var. narutoensis	streptomycin
	eurocidicus	A16316-C
30	fradiae	hybrimycins and neomycins
	fradiae var. italicus	aminosidine

TABLE I (Continued)

Organism	Antibiotic
Streptomyces	
galbus	streptomycin
griseus	streptomycin
griseoflavus	MA 1267
hofuensis	seldomycin complex
hygroscopicus	hygromycins, leucanicidin, and hygrolidin
hygroscopicus forma _glebosus_	glebomycin
hygroscopicus var. _limoneus_	validamycins
hygroscopicus var. _sagamiensis_	spectinomycin
kanamyceticus	kanamycin A and B
kasugaensis	kasugamycins
kasugaspinus	kasugamycins
lavendulae	neomycin
lividus	lividomycins
mashuensis	streptomycin
microsporeus	SF-767
netropsis	LL-AM31
noboritoensis	hygromycins
olivaceus	streptomycin
olivoreticuli var. _cellulophilus_	destomycin A

TABLE I (Continued)

	Organism	Antibiotic
5	poolensis	streptomycin
	rameus	streptomycin
	ribosidificus	SF733
	rimofaciens	destomycin A
	rimosus forma	
10	paromomycinus	paromomycins and catenulin
	spectabilis	spectinomycin
	tenebrarius	tobramycin and apramycin
15		
	Streptoverticillium	
	flavopersicus	spectinomycin

TABLE II

Ansamycin Antibiotic-Producing Organisms

Organism	Antibiotic
Micromonospora	
various species	various ansamycins
Nocardia	
mediterranei	rifamycin
Streptomyces	
collinus	ansatrienes and napthomycins
diastochromogenes	ansatrienes and napthomycins
galbus subsp. griseosporeus	napthomycin B
hygroscopicus	herbimycin
hygroscopicus var. geldanus var. nova	geldamycin
nigellus	21-hydroxy-25-demethyl 25-methylthioproto streptovaricin
rishiriensis	mycotrienes
sp. E/784	actamycin and mycotrienes
sp. E88	mycotrienes
spectabilis	streptovaricins
tolypophorous	tolypomycin

TABLE III

Anthracycline and Quinone Antibiotic-Producing Organisms

	Organism	Antibiotic
5		
	Streptomyces	
	caespitosus	mitomycins A, B, and C
	coelicolor	actinorhodin
10	coeruleorubidicus	daunomycin
	cyaneus	ditrisarubicin
	flavogriseus	cyanocycline A
	galilaeus	aclacinomycin A,
		auramycins, and
15		sulfurmycins
	lusitanus	napthyridinomycin
	peuceticus	daunomycin and
		adriamycin
	violochromogenes	arugomycin
	E	
20		MM 4550, and MM 13902
	cattleya	thienamycin
	chartreusis	SF 1623 and
		cephamycin A and B

TABLE IV

β-Lactam Antibiotic-Producing Organisms

Organism	Antibiotic
Agrobacterium	various β-lactams
Cephalosporium acremonium	penicillins and cephalosporins
Chromobacterium	various β-lactams
Gluconobacter	various β-lactams
Nocardia lactamadurans uniformis	cephamycin C nocardicin
Penicillium chrysogenum	various penicillins and other β-lactams
Serratia	various β-lactams
antibioticus argenteolus	clavulanic acid asparenomycin A,

TABLE IV (Continued)

	Organism	Antibiotic
5	Streptomyces	
	cinnamonensis	cephamycin A and B
	clavuligerus	PA-32413-I, cephamycin C,
		A16886A, penicillins
		cephalosporins,
10	clavulanic	
		acid, and other clavams
	fimbriatus	cephamycin A and B
	flavovirens	MM 4550 and MM 13902
	flavus	MM 4550 and MM 13902
15	fulvoviridis	MM 4550 and MM 13902
	griseus	cephamycin A and B
		and carpetimycin A and B
	halstedi	cephamycin A and B
	heteromorphus	C2081X and
20		cephamycin A and B
	hygroscopicus	deacetoxycephalosporin C
	lipmanii	cephamycin, penicillin N,
		7-methoxycephalosporin C,
		A16884, MM4550, MM13902
25	olivaceus	epithienamycin F,
		MM 4550, and MM 13902
	panayensis	C2081X and
		cephamycin A and B
	rochei	cephamycin A and B
30	sioyaensis	MM 4550 and MM 13902
	sp. OA-6129	OA-6129A
	sp. KC-6643	carpetimycin A
	viridochromogenes	cephamycin A and B
	wadayamensis	WS-3442-D
35		

TABLE V

Macrolide, Lincosamide, and Streptogramin
Antibiotic-Producing Organisms

Organism	Antibiotic
Micromonospora	
rosaria	rosaramicin
Streptomyces	
albireticuli	carbomycin
albogriseolus	mikonomycin
albus	albomycetin
albus var.	
coilmyceticus	coleimycin
ambofaciens	spiramycin and
	foromacidin D
antibioticus	oleandomycin
avermitilis	avermectins
bikiniensis	chalcomycin
bruneogriseus	albocycline
caelestis	M188 and celesticetin
cinerochromogenes	cineromycin B
cirratus	cirramycin
deltae	deltamycins
djakartensis	niddamycin
erythreus	erythromycins
eurocidicus	methymycin
eurythermus	angolamycin
fasciculus	amaromycin

5

10

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25

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TABLE V (Continued)

	Organism	Antibiotic
5	Streptomyces	
	felleus	argomycin and picromycin
	fimbriatus	amaromycin
	flavochromogenes	amaromycin and shincomycins
10		
	fradiae	tylosin
	fungicidicus	NA-181
	fungicidicus var.	
	espinomyceticus	espinomycins
15	furdicidicus	mydecamycin
	goshikiensis	bandamycin
	griseofaciens	PA133A and B
	griseoflavus	acumycin
	griseofuscus	bundlin
20	griseolus	griseomycin
	griseospiralis	relomycin
	griseus	borrelidin
	griseus ssp. sulphurus	bafilomycins
	halstedi	carbomycin and leucanicidin
25	hygroscopicus	tylosin
	hygroscopicus subsp.	
	aureolacrimosus	milbemycins
	kitastoensis	leucomycin A_3 and josamycin
30	lavendulae	aldgamycin
	lincolnensis	lincomycin

TABLE V (Continued)

	Organism	Antibiotic
5	loidensis	vernamycin A and B
	macrosporeus	carbomycin
	maizeus	ingramycin
	mycarofaciens	acetyl-leukomycin, and espinomycin
10	narbonensis	josamycin and narbomycin
	narbonensis var. josamyceticus	leucomycin A$_3$ and josamycin
15	olivochromogenes	oleandomycin
	platensis	platenomycin
	rimosus	tylosin and neutramycin
	rochei	lankacidin and borrelidin
20		
	rochei var. volubilis	T2636
	roseochromogenes	albocycline
	roseocitreus	albocycline
25	spinichromogenes var. suragaoensis	kujimycins
	tendae	carbomycin
	thermotolerans	carbomycin
	venezuelae	methymycins
30	violaceoniger	lankacidins and lankamycin

TABLE VI

Miscellaneous Antibiotic-Producing Streptomyces

	Antibiotic Type	Streptomyces Species	Antibiotic
5			
	amino acid analogues	sp.	cycloserine
10	cyclopentane ring-containing	coelicolor	methylenomycin A
		erythrochromogenes	sarkomycin
		kasugaensis	aureothricin and thiolutin
15		violaceoruber	methylenomycin A
	nitro-containing	venezuelae	chloramphenicol
	polyenes	griseus	candicidin
20		nodosus	amphotericin B
		noursei	nystatin
	tetracyclines	aureofaciens	tetracycline, chlor tetracycline, demethyltetra cycline, and demethylchlortetra cycline
25			
		rimosus	oxytetracycline

TABLE VII

Nucleoside Antibiotic-Producing Organisms

Organism	Antibiotic
Corynebacterium michiganese pv. rathayi	tunicamycin analogues
Nocardia candidus	pyrazofurin
Streptomyces antibioticus	ara-A
chartreusis	tunicamycin
griseoflavus var. thuringiensis	streptoviridans
griseolus	sinefungin
lysosuperificus	tunicamycin

TABLE VIII

Peptide Antibiotic-Producing Organisms

5	Organism	Antibiotic
	Actinoplanes	
	missouriensis	actaplanin
	teichomyceticus	teicoplanin
10		
	Bacillus	
	various species	bacitracin, polymixin, and colistin
15	Nocardia	
	candidus	A-35512 and avoparcin
	lurida	ristocetin
	orientalis	vancomycin
20	Streptomyces	
	antibioticus	actinomycin
	aureus	thiostrepton
	canus	amphomycin
	eburosporeus	LL-AM374
25	haranomachiensis	vancomycin
	pristinaespiralis	pristinamycin
	roseosporus	lipopeptides, such as A21978C
	toyocaensis	A47934
30	virginiae	A41030

TABLE IX

Polyether Antibiotic-Producing Organism

	Organism	Antibiotic
5		
	Actinomadura	
	various species	various polyethers
	oligosporus	A80190
10		
	Dactylosporangium	
	various species	various polyethers
	Nocardia	
15	various species	various polyethers
	Streptomyces	
	albus	A204, A28695A and B,
		and salinomycin
20	aureofaciens	narasin
	bobili	A80438
	cacaoi var.	
	asoensis	lysocellin
	chartreusis	A23187
25	cinnamonensis	monensin
	conglobatus	ionomycin
	eurocidicus var.	
	asterocidicus	laidlomycin
	flaveolus	CP38936
30	gallinarius	RP 30504
	griseus	grisorixin

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TABLE IX continued

	Organism	Antibiotic
5	hygroscopicus	A218, emericid, DE3936, A120A, A28695A and B, etheromycin, and dianemycin
	lasaliensis	lasalocid
10	longwoodensis	lysocellin
	mutabilis	S-11743a
	pactum	A80438
	ribosidificus	lonomycin
	violaceoniger	nigericin
15	Streptoverticillium	
	various species	polyethers

20   The present invention is best exemplified by
transforming antibiotic-producing microorganisms with
genes that code for enzymes that catalyze chemical
reactions governing the conversion of primary metabo-
lites into antibiotics. One such enzyme, macrocin
25 O-methyltransferase, catalyzes the final step in the
biosynthesis of tylosin. Transforming tylosin-producing
microorganisms with a macrocin O-methyltransferase-encoding

gene, designated as _tylF_, results in an improved tylosin biosynthetic pathway because observed are increased levels of the _tylF_ gene product in the transformed cells.

5   Accordingly, the present invention also provides a method for increasing the tylosin or tylosin precursor-producing ability of a tylosin-producing microorganism, which comprises culturing a microorganism which produces tylosin or a tylosin precursor by a
10   biosynthetic pathway, said microorganism being transformed with a DNA cloning vector or portion thereof which contains tylosin or tylosin-precursor biosynthetic gene coding for expression of a rate-limiting enzyme or gene product of the biosynthetic pathway, under condi-
15   tions suitable for cell growth, expression of the tylosin or tylosin-precursor biosynthetic gene and production of the tylosin or tylosin precursor, provided that the culturing process provides an increase in the tylosin- or tylosin precursor-producing ability of the
20   microorganism.

          The present invention utilizes antibiotic biosynthetic genes to increase the antibiotic-producing ability of an organism. A small number of antibiotic biosynthetic genes have been cloned, characterized, and
25   described in the relevant literature. Methods for isolating antibiotic biosynthetic genes have been developed, but one especially preferred method is described in Baltz _et al._, U.S. Patent Application serial number 742,349, filed June 7, 1985, (equivalent
30   to European Pat. Appl. No. 86304239.6, Pub. No. 204,549) which is incorporated by reference. The present tylosin

antibiotic biosynthetic genes used in a specific exem-
plification of the present method initially were isolat-
ed from a λ library constructed in substantial
accordance with the procedure described in Fishman et
5   al., 1985, J. Bacteriology 161:199-206.

A schematic representation of the tylosin
biosynthetic pathway is presented in Figure 1; each
arrow in Figure 1 represents a step which is catalyzed
by one or more tylosin biosynthetic gene products. The
10   gene(s) responsible for each conversion is indicated
above each arrow. Each genotypic designation may
represent a class of genes that contribute to the same
phenotype. A number of expression vectors are used to
exemplify the present invention. These vectors comprise
15   one or more tylosin biosynthetic genes and can be
obtained from the Northern Regional Research Laborato-
ries (NRRL), Peoria, Illinois 61604. Table X provides
a brief description of each of the plasmids used to
exemplify the method of the present invention.

20

Table X

Plasmids Comprising Tylosin Biosynthetic Genes

Host/Designation	Tylosin Gene(s)	NRRL Accession No.	Date of Deposit	Map
E. coli K12 HB101/pHJL280	D, E, F, H, J	B-18043	February 18, 1986	Fig. 2
E. coli K12 HB101/pHJL284	C, F, J	B-18044	February 18, 1986	Fig. 3
E. coli K12 HB101/pHJL309	L, M	B-18045	February 18, 1986	Fig. 4
E. coli K12 HB101/pHJL311	C, F, J, K, H	B-18046	February 18, 1986	Fig. 5
E. coli K12 JM109/pHJL315	D, E, F, H, J	B-18047	February 18, 1986	Fig. 6

A number of Streptomyces fradiae strains are described
which have mutant tylosin biosynthetic genes and,
therefore, make much less tylosin than the strain from
which they were derived. Table XI provides a brief
5 description of these mutant strains.

Table XI

Streptomyces fradiae Mutants Defective in
Tylosin Biosynthesis

Strain Designation	Mutant Gene	ATCC* or NRRL Accession No.	
GS15	tylF	NRRL	18058 (deposited March 19, 1986)
GS16	tylE	ATCC	31664 (publicly available)
GS28	tylF	NRRL	18059 (deposited March 19, 1986)
GS33	tylL		
GS48	tylD	NRRL	12170 (publicly available)
GS52	tylC	NRRL	18060 (deposited March 19, 1986)
GS62	tylM		
GS76	tylD		
GS85	tylH	NRRL	12171 (publicly available)
GS88	tylK		
	tylJ		

*ATCC is the American Type Culture Collection, Rockville, MD 20852, and NRRL is the Northern Regional Research Laboratory, Peoria, IL 61604.

Plasmids pHJL280, pHJL284, and pHJL315 were
used to transform Streptomyces fradiae GS15 and Strepto-
myces fradiae GS28. The GS15 and GS28 strains were
prepared from S. fradiae C4 by nitrosoguanidine mutagen-
5   esis. S. fradiae C4 was derived from S. fradiae T59235
(ATCC 19609) by mutagenesis. The GS15 strain makes
almost no tylosin, and the GS28 strain makes low levels
of tylosin, as compared with the C4 strain. The de-
creased or nonexistent tylosin-producing ability of the
10   GS15 and GS28 strains is believed to result from muta-
tions affecting the tylF gene, which encodes macrocin
O-methyltransferase (MOMT). The MOMT enzyme, which is
required for the conversion of macrocin to tylosin in
the tylosin biosynthetic pathway, is frequently present
15   in reaction rate-limiting amounts in tylosin-producing
strains. Plasmids pHJL280, pHJL284, and pHJL315 remove
this reaction limitation by providing a means for
increasing both the copy number of the tylF biosynthetic
gene and also the concentration of macrocin
20   O-methyltransferase available for tylosin biosynthesis.
Accordingly, fermentation of S. fradiae GS15/pHJL280, S.
fradiae GS15/pHJL284, S. fradiae GS15/pHJL315, S.
fradiae GS28/pHJL284, S. fradiae GS28/pHJL280, and S.
fradiae GS28/pHJL315 for 72 hours results in about a
25   2-fold to a 6-fold increase in the production of
macrocin O-methyltransferase over that produced in the
C4 strain and a 120-fold increase over that produced in
the GS28 strain.

Plasmid pHJL280 was also used to transform:
30   (1) Streptomyces fradiae GS16; (2) S. fradiae GS48; (3)

<u>S</u>. <u>fradiae</u> GS76; and (4) <u>S</u>. <u>fradiae</u> GS88 which produce
tylosin below detection limits and were derived by
mutagenesis of the C4 strain. Untransformed strains
GS16, GS48, GS76, and GS88 respectively produce a

5   defective enzyme or a rate-limiting amount of (1) the
<u>tylE</u>, demethylmacrocin <u>O</u>-methyltransferase, enzyme;
(2) the <u>tylD</u> enzyme, which is required for addition or
biosynthesis of 6-deoxy-D-allose; (3) the <u>tylH</u> enzyme,
which is required for oxidation of the C-23 methyl

10   position of tylactone; and (4) the <u>tylJ</u> enzyme.
Untransformed strains GS16, GS48, GS76, and GS88,
respectively, tend to accumulate demethylmacrocin,
demycinosyl tylosin, 23-deoxydemycinosyl tylosin, and
demycinosyl tylosin rather than the desired tylosin

15   antibiotic compound.

Plasmid pHJL280 provides a means for increas-
ing the efficiency of the tylosin biosynthetic pathway
by not only providing a non-defective gene but also by
increasing the copy number of the <u>tylD</u>, <u>tylE</u>, <u>tylH</u>, and

20   <u>tylJ</u> biosynthetic genes and by increasing the intracel-
lular amount of the products specified by these genes.
The concentration of available <u>tylE</u> gene product,
therefore, is increased, resulting in an elevated
amount of enzyme capable of driving the conversion of

25   demethylmacrocin to macrocin to tylosin in the tylosin
biosynthetic pathway. Similarly, the concentration of
available <u>tylD</u>, <u>tylH</u>, and <u>tylJ</u> gene products is also
increased, resulting in the production of elevated
amounts of the enzymes capable of driving the 6-deoxy-

30   D-allose addition and C-23 oxidation of tylosin

precursors. Fermentation of <u>Streptomyces</u> <u>fradiae</u>
GS16/pHJL280, <u>S</u>. <u>fradiae</u> GS48/pHJL280, <u>S</u>. <u>fradiae</u>
GS76/pHJL280, and <u>S</u>. <u>fradiae</u> GS88/pHJL280 for 144-168
hours results in yields of tylosin that are significant-

5   ly increased over that of the untransformed, low-
tylosin-producing, mutant strains. Such transformed
strains have higher enzyme levels of the particular
enzymes encoded on plasmid pHJL280 than the parent C4
strain and thus further exemplify the present invention.

10   Plasmid pHJL280 can be used to improve the tylosin-
producing ability of any organism in which the <u>tylD</u>,
<u>tylE</u>, <u>tylF</u>, <u>tylH</u>, or <u>tylJ</u> gene products (or any combina-
tion thereof) are present in rate-limiting amounts for
tylosin biosynthesis. .

15   Plasmid pHJL284 was also used to transform
<u>Streptomyces</u> <u>fradiae</u> GS52, a low tylosin-producing,
mutant strain derived from the C4 strain that produces
reaction-limiting amounts of an enzyme required for the
biosynthesis or addition of mycarose to de-<u>O</u>-methyl-

20   lactenocin. Thus, the tylosin biosynthetic pathway of
<u>Streptomyces</u> <u>fradiae</u> GS52 tends to produce desmycosin
rather than the desired tylosin antibiotic compound.
Plasmid pHJL284 provides a means for improving the
synthetic efficiency of this pathway by providing a

25   non-defective biosynthetic gene and by increasing the
copy number of the <u>tylC</u> biosynthetic gene. The concen-
tration of available <u>tylC</u> gene product in the trans-
formed strain, therefore, is increased, resulting in the
elevated production of enzyme capable of driving the

30   desired addition reaction. Accordingly, fermentation of

Streptomyces fradiae GS52/pHJL284 for 144-168 hours
results in a level of tylosin production that is signif-
icantly increased over that of the untransformed mutant
strain and results in higher tylC enzyme levels than
5   those in the parent C4 strain. Plasmid pHJL284 was also
used in the present method to improve the
tylosin-producing ability of Streptomyces fradiae GS88,
a tylJ mutant, and thus can also be used in the present
method to improve the tylosin-producing ability of any
10 organism in which the tylC, tylF, or tylJ gene products
(or any combination thereof) are present in rate-
limiting amounts for tylosin biosynthesis.

        Plasmid pHJL309 contains the tylL and tylM
biosynthetic genes and was used in the present method to
15 improve the tylosin-producing ability of Streptomyces
fradiae GS33, a tylL mutant, and GS62, a tylM mutant.
Plasmid pHJL309 can also be used in the present method
to improve the tylosin-producing ability of any organism
in which the tylL or tylM gene products (or both) are
20 present in rate-limiting amounts for tylosin
biosynthesis.

        Plasmid pHJL311 contains the tylC, tylF, tylH,
tylJ, and tylK biosynthetic genes and so was used in the
present method to improve the tylosin-producing ability
25 of Streptomyces fradiae GS52, a tylC mutant; GS88, a
tylJ mutant; GS15 and GS28, both of which are tylF
mutants; and GS85, a tylK mutant. Plasmid pHJL311 can
also be used in the present method to improve the
tylosin-producing ability of any organism in which the
30 tylC, tylF, tylH, tylJ, or tylK gene products (or any

combination thereof) are present in rate-limiting
amounts for tylosin biosynthesis.

Plasmid pHJL315 contains the tylD, tylE, tylF,
tylH, and tylJ biosynthetic genes and so was used in the
5   present method to improve the tylosin-producing ability
of Streptomyces fradiae GS48, a tylD mutant; GS88, a
tylJ mutant; GS16, a tylE mutant; GS76, a tylD, tylH
double mutant; and GS15 and GS28, both of which are tylF
mutants. Plasmid pHJL315 can also be used in the
10   present method to improve the tylosin-producing ability
of any organism in which the tylD, tylE, tylF, tylH, or
tylJ gene products (or any combination thereof) are
present in rate-limiting amounts for tylosin
biosynthesis.

15   These results demonstrate that the vectors of
the present invention can increase the antibiotic-producing
ability of an antibiotic-producing organism by providing
higher enzyme or other gene product levels, as compared
to an untransformed organism, of an enzyme or other gene
20   product that is rate-limiting in an antibiotic biosyn-
thetic pathway. However, plasmid maintenance in an
antibiotic-producing host cell sometimes requires
significant expenditures of the cell's energy, energy
that might otherwise be used to produce antibiotic.
25   Thus, certain microorganisms transformed with autonomous-
ly replicating vectors actually show a decrease in
antibiotic-producing ability, even though the same
vectors can increase the antibiotic-producing ability of
other organisms. Not wishing the present invention to

be bound or limited in any way by theory, this apparent
anomaly can be explained by the fact that antibiotics
are produced from primary metabolites, such as acetate,
propionate, malonyl-CoA, methylmalonyl-CoA, and glucose,
5   by the action of specific enzymes. These enzymes are
usually not present during the rapid growth phase of an
organism and so do not rob the growing cell of needed
compounds. As growth becomes limited by nutritional
conditions, antibiotic biosynthetic genes are believed
10   to be activated, causing the synthesis of enzymes that
redirect the flow of certain primary metabolites into
antibiotic products.

The synthesis of antibiotics is also believed
to be a dispensable function in antibiotic-producing
15   organisms because mutants blocked in the biosynthesis of
antibiotics are viable and grow as well as the
antibiotic-producing parent. Wild-type strains produce
a relatively small amount of antibiotic, which is
apparently adequate to provide the organism with a
20   selective advantage.

The development of industrial antibiotic
producing strains from natural isolates involves many
cycles of mutation and selection for higher antibiotic
production. Because the synthesis of antibiotics drains
25   primary metabolites and cellular energy away from growth
and maintenance functions, it is believed that selection
for higher antibiotic production frequently occurs at
the expense of the vitality of the organism. Thus, the
generation of high antibiotic-producing strains involves
30   finely balancing the cells nutritional and energy

resources between growth-maintenance functions and
antibiotic production. As a consequence of this fine-
tuning, high-yielding production strains tend to be
extremely sensitive to factors that affect cellular
5 physiology. For example, introduction of autonomously-
replicating vectors, notably multicopy plasmids, some-
times tends to decrease the antibiotic-producing ability
of an organism that normally produces antibiotics at
high levels. The mechanism of this inhibition is not
10 clear, but may occur at an early step in the biosynthe-
sis of the antibiotic because measurable levels of
antibiotic precursors do not accumulate under these
conditions. In addition, autonomously replicating
vectors may drain pools of precursors for DNA or RNA
15 synthesis or, in high copy number, may titrate DNA
binding proteins, such as RNA polymerase, DNA polymer-
ase, polymerase activators, or repressors of gene
expression. Another frequent limitation of autonomously
replicating vectors is spontaneous loss. Spontaneous
20 loss is especially problematical when the vector reduces
growth rate as frequently occurs. Selection for a
resistance marker on the plasmid can ensure the growth
of homogeneous, plasmid-containing populations but can
also disrupt the fine physiological balance (already
25 mentioned) of an antibiotic fermentation. Selection for
unstable plasmids operates by killing or inhibiting the
bacteria that lose the plasmid and can result in a
reduced growth rate.

          The negative effect, sometimes observed, of
30 autonomously replicating vectors on the

antibiotic-producing ability of a microorganism is
greatest in high-producing strains that are delicately
balanced with respect to growth-maintenance functions
and antibiotic production. The present invention
5 overcomes this previously unrecognized problem of the
negative effect of autonomous plasmid replication on
high-producing strains by providing methods of culturing
the transformed host cell to facilitate identification
of transformed cells containing integrated plasmid and,
10 in addition, by providing vectors with features that
also facilitate detection of integration. Selecting a
culturing procedure that results in integration is
important in improving the antibiotic-producing ability
of highly selected and conventionally improved
15 antibiotic-producing organisms. Organisms or strains
that have a low antibiotic-producing ability can be
improved by transformation via either integration or
autonomous vector replication. As those skilled in the
art of fermentation technology will appreciate, the
20 greatest improvement in antibiotic-producing ability is
shown when the present invention is applied to low
antibiotic-producing strains.

Integration of plasmid DNA is readily accom-
plished by transforming a given antibiotic-producing
25 strain or mutant thereof according to standard transfor-
mation procedures, selecting or otherwise identifying
the transformants, and then culturing the cells under
conditions that do not require the presence of plasmid
DNA sequences for the host cell to grow and replicate.
30 After several generations under non-selective

conditions, certain cells will no longer contain free
plasmid DNA, so by selecting for or otherwise identify-
ing plasmid DNA sequences present in the host cell, one
can identify host cells in which the plasmid DNA has

5   integrated into the chromosomal (genomic) DNA of the
cell. This culturing technique to obtain integratic of
vector DNA is especially useful when used in conju. ion
with a vector that is inherently unstable in the trans-
formed host cell, so that culturing without selective

10   pressure to maintain the vector generates segregants
that are free of the plasmid. Bibb et al., 1980, Nature
384:526-531, describe a DNA sequence needed for stable
inheritance of a vector, and a variety of vectors have
been constructed that lack this stability sequence.

15   For instance, cloning vectors pHJL210 and
pHJL401, which were used to construct the plasmids of
the present invention, lack this stability sequence.
Plasmid pHJL210 is disclosed in U.S. Patent Appli tion
Serial No. 639,566, filed August 10, 1984, (equivalent

20   to European Publication No. 176199). Plasmid pHJL401 's
disclosed in U.S. Patent Application Serial No. 841,920,
filed March 20, 1986, which is a continuation-in-part of
Serial No. 763,172, filed August 7, 1985, (equivalent to
European Application No. 86306011.7, filed August 5,

25   1986, Pub. 213,779). As used, "unstable" refers to plasmids
that are lost at high frequency by transformed cells only
when those cells are cultured in the absence of selective
pressure for plasmid maintenance because, for example,
plasmids such as pHJL210 and pHJL401 are quite stable

when selective pressure is applied to the transformed
host cell. When host cells transformed with stable
vectors are cultured in the absence of selective pressure,
the vector is not lost with the high frequency observed

5   with unstable vectors, and identification of integrants
is made difficult by the great number of cells that
still contain autonomously replicating plasmid even
after growth under nonselective conditions. Selection
for integrants is more fully described below. Once the

10   vector DNA has integrated into the chromosomal DNA of
the host cell, one observes the maximum increase in
antibiotic-producing ability for that host cell, because
inhibition by autonomously replicating plasmid no longer
occurs.

15   Integration of vectors containing cloned genes
into the genome of the producing organism can be
achieved in a number of ways. One way is to use a
lysogenic bacteriophage or other phage vector that can
integrate into the genome of the host strain. Another

20   approach is to use a plasmid vector carrying the cloned
genes and to screen for integration of the recombinant
plasmid into the host genome by a single recombination
event between the cloned sequence and the homologous
chromosomal sequence. Integration frequency of a vector

25   can be dramatically increased by adding DNA homologous
to the genomic DNA of the host cell to the vector. As
used "integration" refers both to a single recombination
event, known as Campbell-type recombination, and also to
a double-crossover event, which results in exchange of

30   genetic information between the vector and the

chromosome. With double-crossover recombination, only a portion of the vector integrates into the chromosomal DNA.

5 For example, a plasmid carrying cloned tylosin biosynthetic genes (tyl) could integrate into the Streptomyces fradiae genome by a single crossover between the tyl genes on the plasmid and the homologous tyl genes in the genome. Another option would be to put a non-tyl S. fradiae DNA sequence on the plasmid in

10 addition to the cloned tyl genes and to screen for integration at the locus corresponding to the non-tyl sequence. The latter approach avoids the possible mutagenic effects of integration into the tyl sequences, but if double-crossover recombination is desired, the

15 vector should comprise the antibiotic biosynthetic genes flanked by separate sequences of homologous DNA.

To avoid the potentially adverse effects, however remote, of a recombinant plasmid (either autono- mously replicating or integrated) on tylosin production,

20 one can make use of the ability of Streptomyces fradiae to take up tylosin precursors from the culture medium and convert them to tylosin. In one fermentation of a tylosin-producing strain that had been transformed with plasmid pHJL280 and cultured to obtain integrants, only

25 a subpopulation (~18%) of the cells were thiostrepton resistant, which indicates the presence of plasmid pHJL280 sequences. However, this subpopulation con- tained multiple copies of the genes for two rate- limiting enzymes, demethylmacrocin-O-methyltransferase

30 (DMOMT) and macrocin-O-methyltransferase (MOMT), and

consequently elevated (about 9 fold) levels of the two
enzymes, and was able to convert all of the normally
accumulated demethylmacrocin and macrocin to tylosin
(see Table XIV).

5   Thus, one can develop specific strains of S.
fradiae containing multiple copies of rate-limiting
genes and high enzyme levels to act as converters of
accumulated precursors to tylosin. These converter
strains can be used in several different ways: (1) the
10   converter strain can be co-inoculated into the fermentor
with the normal production strain at a low ratio of
converter:producer; (2) the converter strain can be
introduced into a production fermentation culture late
in the cycle to convert intermediates; (3) the converter
15   strain can be kept in a separate "reactor", to which the
fermentation production broth from the producer strain
would be added; or (4) the converter strain can be
immobilized on a column, and fermentation broth from the
producer strain passed through. Those skilled in the
20   art will recognize that having separate production and
converting populations eliminates the adverse effects
that recombinant plasmids sometimes have on antibiotic
production in high antibiotic-producing strains.

          Separate populations also eliminate vector
25   stability problems, because the converting strains can
be grown in small vessels in which antibiotic selection
or some other selection means for maintenance of the
plasmid can be carefully regulated and controlled. In
essence, the converting strain is a source of enzymes,
30   and the production of these enzymes at high level can be

approached in much the same way as production of pro-
teins from recombinant plasmids in E. coli.

Of course, antibiotic production is only
increased by the method of the present invention when
5   the transforming DNA comprises a gene that encodes the
rate-limiting enzyme of the untransformed strain.
Various methods for determining the rate-limiting step
in the biosynthesis of an antibiotic are known in the
art (Seno and Baltz, 1982, Antimicrobial Agents and
10   Chemotherapy 21:758-763), but there is no need to
identify the rate-limiting step when the entire set of
antibiotic biosynthetic genes are available for intro-
duction into the antibiotic-producing strain. If a
rate-limiting enzyme is not known, the antibiotic-
15   producing strain is transformed with the entire set of
antibiotic biosynthetic genes, thus ensuring that, no
matter what enzyme is rate-limiting, the transformed
host cell will have higher levels of the rate-limiting
enzyme than the untransformed host cell. Often, howev-
20   er, the rate-limiting enzyme of an antibiotic biosyn-
thesis pathway will be known, and the method of the
present invention can be used to increase the
antibiotic-producing ability of the organism by trans-
forming the organism with a vector that encodes the
25   rate-limiting antibiotic biosynthetic enzyme.

For instance, the GS15 strain, which produces
no readily detectable tylosin (the level of tylosin
produced by these cells is below the detection limits
for the assay used to determine tylosin levels) and the
30   GS28 strain, which produces very low levels of tylosin,

contain tylF mutations, so that it is a relatively
simple matter to identify the rate-limiting step in
tylosin biosynthesis in these mutant strains. The
strain from which the GS15 and GS28 strains were de-

5   rived, designated Streptomyces fradiae C4, produces high
levels of tylosin and accumulates relatively large
amounts of macrocin, the immediate precursor of tylosin
on which the tylF gene product acts to form tylosin.
Other S. fradiae strains that produce even more tylosin

10   than the C4 strain accumulate even more macrocin than
the C4 strain. These observations indicate that the
tylF gene product is present in rate-limiting amounts
for the biosynthesis of tylosin in high
tylosin-producing strains. Transformation of these

15   macrocin-accumulating strains with a vector comprising
the tylF gene followed by isolation of those
transformants that only contain integrated copies of the
vector yields transformants that produce more tylosin
than the untransformed cells. The increase in tylosin

20   production observed in these transformants is related to
the amount of macrocin that accumulates in the
untransformed cells. It will be apparent to those
skilled in the art that the transformants produced by
the foregoing procedure might still contain

25   rate-limiting amounts of the tylF gene product, in which
case a further increase of the tylF copy number would
further increase tylosin yield, or the transformed
strains might now contain rate-limiting amounts of yet
another antibiotic biosynthetic enzyme, the level of

which could be made non-rate-limiting by the method of
the present invention.

The present invention provides both a method
and recombinant DNA cloning vectors for increasing the
5 production of an antibiotic by manipulation of antibiot-
ic biosynthetic pathways. An illustrative antibiotic
biosynthetic pathway involves the biosynthesis of
tylosin, a complex macrolide produced by strains of
Streptomyces fradiae, Streptomyces rimosus, and Strepto-
10 myces hygroscopicus. Tylosin is composed of a 16-member
branched lactone (tylonolide) to which three sugars
(mycarose, mycaminose, and mycinose) are attached. The
lactone is derived from two acetates, five propionates,
and a butyrate by condensation of a propionyl-S-coenzyme
15 A molecule with two malonyl-S-coenzyme A molecules, four
methylmalonyl-S-coenzyme A molecules, and an
ethylmalonyl-S-coenzyme A molecule by a scheme believed
analogous to that involved in fatty acid biosynthesis.
Lactone formation, sugar biosynthesis/attachment, and
20 the conversion of resultant intermediate compounds to
tylosin are catalyzed by a series of gene-encoded
enzymes. Cloning genes that code for such enzymes
allows modification and improvement in the operational
efficiency of the tylosin biosynthetic pathway and is
25 illustrative of the present invention.

Illustrative tylosin biosynthetic genes that
can be used for purposes of the present invention
include, for example, the tylC, tylD, tylE, tylF, tylH,
tylJ, tylK, tylL, and tylM, genes. Of this group, the
30 tylF gene is preferred because the macrocin

O-methyltransferase enzyme encoded thereby appears to be
rate-limiting in the tylosin biosynthetic pathway of
most tylosin-producing strains. Macrocin accumulates to
unacceptable levels under conditions of optimum fermen-

5   tation of Streptomyces fradiae because of the rate-
limiting steps catalyzed by the tylF gene product. The
tylF enzyme catalyzes the conversion of macrocin to
tylosin, as depicted in Figure 1 of the accompanying
drawings. Over-production of the tylF gene product,

10   macrocin O-methyltransferase, results in the more
efficient operation of the tylosin biosynthetic pathway
as indicated by increased antibiotic yield and lower
cost of fermentation.

Those skilled in the art will recognize that

15   the present invention is not limited to the use of
plasmids pHJL280, pHJL284, pHJL309, pHJL311, or pHJL315.
The antibiotic biosynthetic genes contained in these
vectors can be excised in whole or in part and ligated
into any number of different recombinant DNA cloning

20   vectors. For instance, digestion of plasmid pHJL280
with restriction enzymes BamHI and BglII yields five
BamHI-BamHI fragments with sizes of ∿10.3 kb, ∿6.54 kb,
∿2.3 kb, ∿1.7 kb, and ∿1.0 kb; two BamHI-BglII fragments
with sizes of ∿2.9 kb and 2.0 kb; and one BglII-BglII

25   fragment ∿0.2 kb in size. The ∿2.9 kb BamHI-BglII
fragment of plasmid pHJL280 contains the tylF gene.
Digestion of plasmid pHJL280 with restriction enzymes
BglII and EcoRI generates four fragments: an ∿11.24 kb
EcoRI-EcoRI fragment; an ∿11.5 kb BglII-EcoRI fragment;

30   an ∿4.0 kb EcoRI-BglII fragment, and an ∿0.2 kb

BglII-BglII fragment. The ~4.0 kb EcoRI-BglII fragment
of plasmid pHJL280 contains the tylE gene.

        Digestion of plasmid pHJL284 with restriction
enzymes BamHI and EcoRI generates three BamHI-BamHI
5   fragments with sizes of ~9.7 kb, ~2.3 kb, and ~1.0 kb;
·   and four EcoRI-BamHI fragments with sizes of ~6.24 kb,
~4.3 kb, ~2.3 kb, and ~1.1 kb. The ~2.3 kb BamHI-EcoRI
fragment of plasmid pHJL284 contains the tylF gene.
Digestion of plasmid pHJL284 with restriction enzyme
10   EcoRI generates two fragments with sizes of ~16.4 kb and
~10.54 kb; the ~16.4 kb fragment contains the tylF,
tylC, and tylJ genes. The ~1.7 kb EcoRI-BamHI restric-
tion fragment of plasmid pHJL311 comprises the tylK
gene. The ~18.5 kb EcoRI restriction fragment, as well
15   as the ~8.3 kb BamHI-KpnI restriction fragment, of
plasmid pHJL309 contains the tylL and tylM genes.

        Any of the aforementioned tyl gene-containing
fragments can be ligated into other vectors to make
vectors useful in the present method. Such other
20   vectors include, for example, those vectors disclosed in
U.S. Patent Nos. 4,468,462; 4,513,086; 4,416,994;
4,503,155; and 4,513,185; and also plasmids pIJ101,
pIJ350, pIJ702 (ATCC 39155), SCP2* (NRRL 15041),
pHJL192, pHJL197, pHJL198, pHJL210, pHJL211, pHJL400,
25   pHJL401, pHJL302, pIJ922, pIJ903, pIJ941, pIJ940, and
pIJ916. These vectors replicate in Streptomyces fradiae
and other tylosin-producing strains and are thus useful
for cloning the present antibiotic biosynthetic genes.
The "unstable" vectors described above are preferred
30   when integration of the vector is desired.

Illustrative Streptomyces strains that can be
used for purposes of the present invention include, for
example, S. fradiae, S. fradiae GS52, S. fradiae GS48,
S. fradiae GS16, S. fradiae GS28, S. fradiae GS15, S.
5   fradiae GS76, S. rimosus, and S. hygroscopicus. Strep-
tomyces hygroscopicus and S. rimosus are well known,
having been deposited at the American Type Culture
Collection (ATCC), Rockville, Maryland 20852. A number
of strains of S. hygroscopicus can be obtained under the
10   accession numbers ATCC 27438, ATCC 21449, ATCC 15484,
ATCC 19040, and ATCC 15420, and S. rimosus can be
obtained under the accession number ATCC 10970. Of the
Streptomyces taxa, S. fradiae GS16, S. fradiae GS15, and
S. fradiae GS28 are preferred, especially for transfor-
15   mation with plasmid pHJL280. Streptomyces fradiae is
also an especially well known microorganism and several
strains are available, on an unrestricted basis, from
the Northern Regional Research Laboratory (NRRL),
Peoria, Illinois 61604 and the ATCC under the respective
20   accession numbers NRRL 2702, NRRL 2703, and ATCC 19609.

The recombinant plasmids described in the
present invention each comprise one or more antibiotic
biosynthetic genes. Unless part of a polycistron, each
antibiotic biosynthetic gene normally comprises: (1) a
25   promoter that directs transcription of the gene; (2) a
sequence that, when transcribed into mRNA, directs
translation of the transcript; (3) a protein-coding
sequence; and (4) a transcription terminator. Each of
these elements is independently useful and can, through
30   the techniques of recombinant DNA technology, be used to

form recombinant genes of great variety. As one exam-
ple, the protein-coding sequence for the tylF gene can
be linked to the promoter, translation-activating
sequence, and transcription-terminating sequence from a

5   non-Streptomyces fradiae gene to form a recombinant gene
that functions in the host from which the non-S. fradiae
sequences were isolated. Such a novel gene could be
used to produce a hybrid antibiotic if introduced into
an organism that produced an antibiotic or antibiotic

10   intermediate that is not found in the tylosin pathway
but that would serve as a substrate for the novel gene
product. Similarly, the promoter and other regulatory
elements of the tylF gene could be linked to the coding
sequence of a non-tylosin antibiotic biosynthetic gene

15   to prepare a hybrid gene that would function in S.
fradiae. Thus, the individual elements of each of the
antibiotic biosynthetic genes on each of the plasmids
described herein comprise an important component of the
present invention.

20   For example, sequence data on the tylF nucleo-
tide sequence has identified the tylF promoter, which
comprises an important aspect of the present invention.
The sequence (only one strand of which is depicted for
convenience) is shown below; the promoter and

25   translation-activating sequence of the tylF gene are
believed to reside in the sequence between residues 1

and 207. The sequence ends with the beginning of the
coding region of the tylF gene.

As an additional embodiment, the gene sequence for
the tylF gene is provided. In particular, the entire tylF gene,
including the promoter and translation-activating sequence noted
above, has been found to have the following sequence:

5

```
                10        20        30        40
    5'-TTC GCG GGA TGG ATG CTG ACC CGG GGG TCG GCC AGC AGC GCC CGG ACG

10      50        60        70        80        90
        TGA TCT GGC GGG AGA TCA GCC AGA CCG GCG CCC CGT CCC ACA GCT CGG

        100       110       120       130       140
        CCC GGG CGA TCG GCT CCT CCG CCC GGA GGG CGG CGT ACT GCT CGG GAG
15
        150       160       170       180       190
        GGC TGA AGG GAC AGG TGC GGG CGA CCG GCC AGG CGA TGC TGC GCC GGC

                200       210       220       230       240
20      CTG CGG CCC CGT CGG TGT CGT TGG CGC GTG CTG CGG GCA ACA GAA TCC

                250       260       270       280
        CCT TTT GTG ACG GGC GGG CGT CCC CGG ACG AGG ACA CGA CTC GCT GCG

25      290       300       310       320       330
        GCC TCA ACG AAA ACA CCG TGT CCG GTG CCC AGG CCA CGA ACG GTG ACC

        340       350       360       370       380
        GGT CTG TGT CAG GTC GCC CGT GGT GAC GGG CTC CGG GGC GGC GGC GCG
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           390           400           410           420           430
      GGC GGC CGA CCT TGA CAT ACC CGC GGC CGG GCT CCT CGT TCC GGC GCG


5                440           450           460           470           480
      GCC CGC GCC GAT AGC GTC CGT CCT CAC CGG CTC CGG CGT CCG CGT CCC


                 490           500           510           520
      CGC CGG GAC GTG CCA CCT CTC CCG ACC CCG CGA GCC GAT CGA CCC GCT

10
      530           540           550           560           570
      ACT GGA GGA CCC GTG GCA CCT TCC CCG GAC CAC GCC CGC GAT CTC TAC
                      VAL ALA PRO SER PRO ASP HIS ALA ARG ASP LEU TYR
                                       5                    10

15
          580           590           600           610           620
      ATC GAG CTG CTG AAG AAG GTC GTC TCG AAC GTC ATC TAC GAG GAC CCC
      ILE GLU LEU LEU LYS LYS VAL VAL SER ASN VAL ILE TYR GLU ASP PRO
              15                    20                    25

20
          630           640           650           660           670
      ACC CAT GTG GCG GGG ATG ATC ACC GAC GCG TCG TTC GAC CGG ACG TCC
      THR HIS VAL ALA GLY MET ILE THR ASP ALA SER PHE ASP ARG THR SER
          30                    35                    40

25
          680           690           700           710           720
      CGT GAG AGC GGC GAG GAC TAC CCC ACG GTC GCC CAC ACG ATG ATC GGC
      ARG GLU SER GLY GLU ASP TYR PRO THR VAL ALA HIS THR MET ILE GLY
      45                    50                    55                    60
```

```
               730          740          750          760
        CTC AAG CGT CTG GAC AAT CTC CAC CGG TGC CTC GCG GAC GTC GTG GAG
        LEU LYS ARG LEU ASP ASN LEU HIS ARG CYS LEU ALA ASP VAL VAL GLU
  5                          65           70           75


               770          780          790          800          810
        GAC GGC GTC CCC GGT GAC TTC ATC GAG ACC GGG GTG TGC CGC GCG CCG
        ASP GLY VAL PRO GLY ASP PHE ILE GLU THR GLY VAL CYS ARG ALA PRO
 10                          80           85           90


               820          830          840          850          860
        TGC ATC TTC GCC CGC GGA CTG CTG AAC GCG TAC GGC CAG GCC GAC CGC
        CYS ILE PHE ALA ARG GLY LEU LEU ASN ALA TYR GLY GLN ALA ASP ARG
 15                 95               100          105


               870          880          890          900          910
        ACC GTC TGG GTC GCC GAC TCC TTC CAG GGC TTT CCC GAG CTG ACC GGG
        THR VAL TRP VAL ALA ASP SER PHE GLN GLY PHE PRO GLU LEU THR GLY
 20                 110              115          120


               920          930          940          950          960
        TCC GAC CAC CCG CTG GAC GTC GAG ATC GAC CTC CAC CAG TAC AAC GAG
        SER ASP HIS PRO LEU ASP VAL GLU ILE ASP LEU HIS GLN TYR ASN GLU
 25     125                  130          135              140


               970          980          990          1000
        GCC GTG GAC CTG CCC ACC AGC GAG GAG ACC GTG CGG GAG AAC TTC GCC
        ALA VAL ASP LEU PRO THR SER GLU GLU THR VAL ARG GLU ASN PHE ALA
 30                          145          150              155
```

```
       1010          1020          1030          1040          1050
   CGG TAC GGG CTG CTC GAC GAC AAC GTC CGT TTC CTG GCG GGG TGG TTC
   ARG TYR GLY LEU LEU ASP ASP ASN VAL ARG PHE LEU ALA GLY TRP PHE
                    160            165            170


       1060          1070          1080          1090          1100
   AAG GAC ACC ATG CCG GCT GCG CCC GTG AAG CAG CTC GCG GTG ATG CGC
   LYS ASP THR MET PRO ALA ALA PRO VAL LYS GLN LEU ALA VAL MET ARG
                    175            180            185


       1110          1120          1130          1140          1150
   CTG GAC GGC GAC TCC TAC GGC GCC ACC ATG GAT GTG CTC GAC AGC CTG
   LEU ASP GLY ASP SER TYR GLY ALA THR MET ASP VAL LEU ASP SER LEU
                    190·           195            200


              1160          1170          1180          1190          1200
   TAC GAG CGG CTG TCG CCG GGC GGT TAC GTC ATC GTC GAC GAC TAC TGC
   TYR GLU ARG LEU SER PRO GLY GLY TYR VAL ILE VAL ASP ASP TYR CYS
   205                 210            215            220


              1210          1220          1230          1240
   ATC CCG GCC TGC CGC GAG CGG TGC ACG ACT TCC GCG ACC GGC TCG GCA
   ILE PRO ALA CYS ARG GLU ARG CYS THR THR SER ALA THR GLY SER ALA
                    225            230            235


       1250          1260          1270          1280          1290
   TCC GCG ACA CGA TCC ACC GGA TCG ACC GCC AGG GCG CTA TTG GCG GCA
   SER ALA THR ARG SER THR GLY SER THR ALA ARG ALA LEU LEU ALA ALA
                    240            245            250
```

```
         1300        1310        1320        1330        1340
    CAG CGG CTG AGT CGT TCC GCC CGA GAG CCC GAC GAG AGC AGG AGA TAT
    GLN ARG LEU SER ARG SER ALA ARG GLU PRO ASP GLU SER ARG ARG TYR
5           255                 260                 265


         1350        1360        1370        1380        1390
    GCG AGA CAC GAC GCG CCC GCT CGG CAT TGA GGG AGC GTG GGT GAT CCA
    ALA ARG HIS ASP ALA PRO ALA ARG HIS
10          270                 275


          1400        1410        1420        1430        1440
    GCC GGA GAT CCA TCC GGA CCG GCG CGG CGA GTT CCA CGC GTG GTT CCA


15            1450        1460        1470        1480
    GAG CCA GCC GAG TTC CGG CGG CTG ACC GGT CAC TCC TTC TCC GTG CCG


       1490        1500        1510        1520        1530
    CAG GTC GTC AAT ATC GCG TGT CCC GGA AAG GCG CCG CTG CGG CAT CCA
20


          1540        1550        1560        1570        1580
    CTT CTG CCG AGG TGC CAC CGG GCC GAG GCC AAG TAC AGC GGC GTG TGT


           1590        1600        1610        1620        1630
25  GCA GGG CGC CGG TGT CGA GGT CGT CGT CGA CGC GCC GGT GTC GAG GTC


            1640
    GTC GTC GAC-3'
```

30

wherein A is deoxyadenyl residue; G is a deoxyguanyl residue; C is a deoxycytidyl residue; and T is a deoxythymidyl residue. The structural gene, as indicated above, begins at residue 541 and continues through to residue 1371, terminating with the stop codon located at residue 1372. The amino

5   acid sequence of the tylF structural gene is that indicated under the corresponding nucleotide sequence. As those skilled in the art will recognize, because of the degeneracy of the genetic code, equivalent sequences to that specifically provided above can be obtained which will encode the same tylF gene product. The means for obtaining such

10   equivalent sequences will be familiar to those skilled in the art. Further, that a specific sequence is provided is not to be construed as limiting the invention in any way.

Streptomyces fradiae strains can be cultured in a number of ways using any of several different

15   media. Carbohydrate sources that are preferred in a culture medium include, for example, molasses, glucose, dextran, and glycerol, and nitrogen sources include, for example, soy flour, amino acid mixtures, and peptones. Nutrient inorganic salts are also incorporated into the

20   medium and include the customary salts capable of yielding sodium, potassium, ammonium, calcium, phosphate, chloride, sulfate, and like ions. As is necessary for the growth and development of other microorganisms, essential trace elements are also added.

25   Such trace elements are commonly supplied as impurities

incidental to the addition of other constituents of the
medium. S. fradiae strains are grown under aerobic
culture conditions over a relative wide pH range of
about 5.5 to 8 at temperatures ranging from about 25° to
5   37°C. In particular, tylosin can be produced by culti-
vation of tylosin-producing strains of, for example, S.
fradiae such as those containing the vectors provided by
the present invention. The culture medium employed can
be any one of a number of media since the organism is
10   capable of utilizing many energy sources. However, for
economy of production, maximum yields of antibiotic, and
ease of isolation of the antibiotic, certain culture
media are preferable. The media which are useful in the
production of tylosin include an assimilable source of
15   carbon such as glucose, sucrose, fructose, starch,
glycerine, molasses, dextrin, brown sugar, corn steep
solids, and the like. The preferred sources of carbon
are glucose and starch. Additionally, employable media
include a source of assimilable nitrogen such as linseed
20   meal, tankage, fish meal, cotton seed meal, oatmeal,
ground wheat, soybean meal, beef extract, peptones (meat
or soy), casein, amino acid mixtures, and the like.
Preferred sources of nitrogen are soybean meal, casein,
and corn steep solids.
25   Mineral salts, for example, those providing
sodium, potassium, ammonium, calcium, magnesium, cobalt,
sulfate, chloride, phosphate, carbonate, acetate, and
nitrate ions, and a source of growth factors such as
distillers' solubles and yeast extract, can be incorpo-
30   rated into the media with beneficial results.

As is necessary for the growth and development
of other microorganisms, essential trace elements should
also be included in the culture medium for growing the
microorganisms employed in this invention. Such trace
5   elements are commonly supplied as impurities incidental
to the additional of the other constituents of the
medium.

The initial pH of the culture medium can be
varied widely. However, it has been found that the
10   initial pH of the medium desirably is between about pH
5.5 and about pH 8.0, and preferably is between about pH
6.5 and about pH 7.0. As has been observed with other
organisms, the pH of the medium gradually increases
throughout the growth period of the organism during
15   which time tylosin is produced, and may attain a pH from
about pH 7.2 to about pH 8.0 or above, the final pH
being dependent at least in part on the initial pH of
the medium, the buffers present in the medium, and the
period of time the organism is permitted to grow.

20   Submerged, aerobic cultural conditions are the
conditions of choice for the production of large amounts
of tylosin. For preparation of relatively small
amounts, shake flasks and surface culture in bottles can
be employed, but for the preparation of large amounts,
25   submerged aerobic culture in sterile tanks is preferred.
The medium in the sterile tank can be inoculated with a
sporulated suspension. However, because of the growth
lag experienced when a sporulated suspension is used in
the inoculum, the vegetative form of the culture is
30   preferred to avoid the pronounced growth lag, thereby

permitting a more efficient use of the fermentation
equipment. Accordingly, it is desirable first to
produce a vegetative inoculum of the organisms by
inoculating a relatively small quantity of culture
5   medium with the spore form of the organism, and when a
young, active, vegetative inoculum has been obtained, to
transfer the vegetative inoculum aseptically to the
large tank. The medium in which the vegetative inoculum
is produced can be the same or a different medium than
10   that utilized for the large scale production of tylosin.

The organisms grow best at temperatures in a
range of about 25°C to about 37°C. Optimal tylosin
production appears to occur at a temperature of about
26-30°C.

15   As is customary in submerged culture process-
es, sterile air is blown through the culture medium.
For efficient growth of the organism and tylosin produc-
tion, the volume of air employed in the tank production
of tylosin preferably is upwards of 0.1 volume of air
20   per minute per volume of culture medium. Efficient
growth and optimal yields of tylosin are obtained when
the volume of air used is at least one volume of air per
minute per volume of culture medium.

The concentration of tylosin activity in the
25   culture medium can readily be followed during the
fermentation period by testing samples of the culture
medium for their inhibitory activity against the growth
of an organism known to be inhibited in the presence of
tylosin.

In general, after inoculation, maximum
production of tylosin occurs within about 2 to 7 days
when submerged aerobic culture or shake flask culture is
employed, and within about 5 to 10 days when surface
5   culture is used.

    If desired, the mycelium and undissolved
solids are removed from the fermentation broth by
conventional means such as filtration or centrifugation.
If desired, the tylosin is removed from the filtered or
10   centrifuged broth by employing adsorption or extraction
techniques familiar to those skilled in the art.

    For the extraction of tylosin from the fil-
tered broth, water-immiscible, polar, organic solvents
are preferred, such including esters of fatty acids, for
15   example, ethyl acetate and amyl acetate; chlorinated
hydrocarbons, for example, chloroform ethylene dichlo-
ride, and trichloroethylene; water-immiscible alcohols,
for example, butyl and amyl alcohols; water-immiscible
ketones, for example, methyl isobutyl ketone and methyl
20   amyl ketone; and others, for example, diethyl ether and
methyl propyl ether. Other solvents of similar charac-
ter can also be employed. Chloroform and amyl acetate
are the presently preferred extraction solvents.

    For the recovery of tylosin by adsorption
25   techniques, various absorbants and ion exchange resins
can be used, for example, carbon, silica gel, alumina,
and ion exchange resins of acidic character such as "XE"
64 and "IRC" 50 (weakly acidic cation exchange resins
sold by Rohm & Haas Company), carboxymethyl cellulose
30   resin, and "Dowex" 50 (a strongly acidic cation exchange

resin sold by The Dow Chemical Company). The tylosin
can be adsorbed on one of the above or similar adsor-
bents from a solution in chloroform, acetone, benzene or
other suitable solvents. The adsorbed tylosin can then
5 be suitable solvents. The adsorbed tylosin can then be
eluted from the adsorbent by suitable elution techniques
such as by washing the adsorbent on which the tylosin is
adsorbed, with a lower alcohol, for example, methanol or
ethanol, or with a lower alcohol containing up to about
10 50 percent of a lower ketone, for example, acetone.
       The organic solvent extract obtained by the
preferred extraction method can be directly evaporated
to dryness to provide crude tylosin. Alternatively the
organic solvent extract can be used to provide purified
15 tylosin by concentration in vacuo the organic solvent
extract of tylosin by decolorizing the concentrate with
carbon, and by precipitating the tylosin by the addition
of a non-polar solvent, for example, petroleum ether.
The precipitate which is thus obtained is a solid,
20 purified tylosin which is usually amorphous. The
amorphous precipitate can be crystallized by employing
one of the crystallizing solvents mentioned above.
Alternatively, tylosin can be recovered from a
tylosin-containing organic extract, by adsorption
25 chromatography, and by recovery of the absorbed tylosin
from the absorbent by elution.
       Other means for preparing the desired product
from the culture medium will be recognized by those
skilled in the art.

The acid addition salts of tylosin can be
formed with mineral acids, for example, sulfuric,
hydrochloride and nitric acid, and with organic acids,
for example, tartaric, gluconic, oxalic and acetic acid.
5 The acid addition salts can be prepared by dissolving
the free base of tylosin in a solvent in which it is
soluble, such as acetone or ether, and adding to the
solution an equimolar amount of the appropriate acid.
The salt which is formed usually precipitates out of
10 solution. In the event the salt does not precipitate,
it can be recovered by evaporating the solution to a
smaller volume to permit precipitation, or by adding a
miscible solvent in which the salt is not soluble.
The following non-limited are provided to
15 further illustrate the invention. Sources of reagents
are provided merely for convenience and in no way limit
the invention.

Example 1

20

Isolation of Plasmid pHJL280

A. Culture of E. coli K12 HB101/pHJL280

25   Lyophils of E. coli K12 HB101/pHJL280 can be
obtained from the NRRL under the accession number NRRL
B-18043. The lyophilized cells are streaked onto L-agar
plates (L agar contains 10 g of Bacto Tryptone, 5 g of
Bacto Yeast Extract, 10 g of NaCl, 2 g of glucose, and

15 g of agar per liter) containing 50 µg/ml ampicillin
to obtain a single-colony isolate of E. coli K12
HB101/pHJL280. One such colony was used to inoculate
100 ml of L broth (L broth is L agar without the agar),
5   which was then incubated aerobically at 37°C overnight
(about 16 hours). The following morning, the cells were
harvested by centrifugation at 10,000Xg for 10 minutes.
The ~1 g of cells obtained by this procedure are used to
prepare plasmid pHJL280 DNA in substantial accordance
10   with the procedure described below.

B.   Plasmid Isolation

        The cell pellet obtained in Example 1A was
15   resuspended in 10 ml of a solution composed of 25%
sucrose and 50 mM Tris-HCl at a pH = 8.0. About 1 ml of
a 10 mg/ml solution of lysozyme in 50 mM Tris-HCl at a
pH = 8.0 was added to the cell suspension, and the
resulting mixture was incubated on ice for 5 minutes.
20   About 4 ml of 0.25 M EDTA, pH = 8.0, were then added to
the cell suspension, and incubation on ice was continued
for another 5 minutes. About 16 ml of lysis solution
(lysis solution contains 0.4% deoxycholate; 1% Brij58
(Sigma Chemical Co., P.O. Box 14508, St. Louis, MO
25   63178); 0.05 M Tris-HCl, pH = 8.0; and 0.0625 M EDTA)
were added to the lysozyme-treated cells, and the
resulting mixture was incubated at 37°C for 15 minutes.
        The cell lysate was cleared by centrifugation
at 48,000Xg for 25 minutes. The supernatant was decant-
30   ed into a separate tube, to which was added 0.1 volume

of 3.0 M NaOAc at a pH = 8.0 and 0.64 volume of isopro-
pyl alcohol. The DNA precipitate was collected by
centrifugation at 20,000Xg for 10 minutes and then
redissolved in 0.1 volume of TE buffer (10 mM Tris-HCl,

5   pH = 7.8, and 1 mM EDTA). The solution of DNA was
incubated at 65°C for 30 minutes and then purified by
equilibrium-density-gradient ultracentrifugation in CsCl
and propidium diiodide. The plasmid pHJL280 DNA ob-
tained by this procedure was dissolved in TE buffer at a

10 concentration of about 1 µg/µl. A restriction site map
of plasmid pHJL280 is presented in Figure 2 of the
accompanying drawings.

Example 2

15

Isolation of Plasmids pHJL284, pHJL309,
pHJL311, and pHJL315

Lyophils of the E. coli strains harboring

20 plasmids pHJL284, pHJL309, pHJL311, and pHJL315 can be
obtained from the NRRL under the accession numbers
listed in Table X. The desired plasmids are each
obtained and purified from the lyophilized cells in
substantial accordance with the teaching of Example 1.

25 Restriction site maps of the plasmids are presented in
Figures 2-6 of the accompanying drawings.

Example 3

Construction of Streptomyces fradiae GS28/pHJL280

5   A culture of Streptomyces fradiae GS28 was
inoculated into 20 ml of trypticase-soya broth (TSB) and
incubated in a water-bath incubator at 29°C at 260 rpm
overnight (about 16 hours). The culture was homogenized
using a homogenizing vessel (Thomas Scientific,

10   Swedesboro, NJ) and a T-Line laboratory stirrer and then
fragmented using a Sonifier Cell Disruptor (Heat Systems
Ultrasonics, Inc.) for 7 seconds at 76 Watts. Four ml
of the homogenized, fragmented culture were inoculated
into 20 ml of TSB (BBL) containing 0.3% weight by volume

15   glycine, and the culture was again incubated overnight
at 29°C. The following morning, the culture was homoge-
nized and recultured as described above. After this
third overnight incubation, the culture was homogenized,
collected, and then washed twice with P media. P media

20   was prepared by adding 103 g of sucrose to 0.25 g of
K_2SO_4 and 2.03 g of $MgCl_2-6H_2O$ and then adding deionized
water to a final volume of 700 ml. The mixture was then
sterilized, and to each 70 ml of solution, about 10 ml
each of 0.05 g KH_2PO_4/100 ml of deionized water; 2.78 g

25      $CaCl_2$/100 ml of deionized water; and 0.25 M TES
(2-([tris-(hydroxymethyl)methyl]-amino)ethanesulfonic
acid)) at a pH = 7.2 were added.

          The cell pellet was resuspended in 15 ml of P
media containing 1 mg/ml lysozyme (Calbiochem, La Jolla,

30   CA 92037) and then incubated at room temperature for
about one-and-one-half hours to form protoplasts. The
protoplasts were gently collected by centrifugation,
washed twice with P media, resuspended in 2 ml of P
media, and incubated on ice until use. About 1 μg of

plasmid pHJL280 DNA was added to about 50 µl of 1 mg/ml
heparin sulfate (Sigma) and incubated on ice for about
10 minutes. Much less plasmid DNA, about 5-100 nano-
grams, can be used to transform Streptomyces fradiae if

5   prepared from a S. fradiae host. The procedure for
isolating Streptomyces plasmid DNA is described in
Hopwood et al., 1985, Genetic Manipulation of Strepto-
myces: A Laboratory Manual (John Innes Foundation,
Norwich, England). The DNA/heparin solution was first

10   added to about 200 µl of protoplasts, and about 0.9 ml
of a solution composed of 55% PEG 1000 (Sigma) in P
medium was then added to the DNA/protoplast mixture, and
the resulting mixture was gently mixed at room
temperature.

15   The mixture was plated in varying aliquots
onto R2 plates using 4 ml of soft-R2-agar overlays. R2
plates contain 30 ml of R2 media and have been dried at
37°C for about 4 days. R2 media is prepared by adding
103 g sucrose, 0.25 g K_2SO_4, 2 ml of trace element

20   solution, 10.12 g $MgCl_2-6H_2O$, 10.0 g of glucose, 2.0 g
of L-asparagine, 0.1 g of Casamino acids, and 22 g of
agar to 700 ml of water; sterilizing the resulting
solution; and finally, adding 100 ml of each of the
following solutions: 0.05 g KH_2PO_4/100 ml of deionized

25   water; 2.22 g $CaCl_2$/100 ml of deionized water; and
0.25 M TES, pH = 7.2. The pH of the final solution is
adjusted to equal 7.2. Trace element solution contains
40 mg $ZnCl_2$, 200 mg $FeCl_3-6H_2O$, 10 mg $CuCl_2-2H_2O$, 10 mg
$MnCl_2-4H_2O$, 10 mg $Na_2B_4O_7-10H_2O$, and 10 mg

30     $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ per liter. The soft-R2-agar overlays

are prepared by adding 51.5 g of sucrose, 5.06 g
$MgCl_2$-$6H_2O$, 1.11 g $CaCl_2$, 50 ml of 0.25 M TES at a pH =
7.2, and 2.05 g agar to enough deionized water to
achieve a final volume of 500 ml. The mixture is

5   steamed to melt the agar, decanted into 4 ml aliquots,
and autoclaved prior to use. After the transformed ·
protoplasts had been plated, the plates were incubated
at 29°C for 24 hours, and then, 4 ml of soft-R2 agar
containing 25 µl of 50 mg/ml thiostrepton (E. R. Squibb,

10   Princeton, NJ 08540) were spread over the protoplasts.
Incubation of the plates at 29°C was continued until
regeneration was complete, usually a period of about
7-14 days, to select for the desired S. fradiae
GS28/pHJL280 transformants.

15   The Streptomyces fradiae GS28/pHJL280 strain
was cultured and produced macrocin O-methyltransferase
and tylosin at levels above that produced in the
untransformed S. fradiae GS28 strain. Macrocin
O-methyltransferase activity was assayed and determined

20   in substantial accordance with the teaching of Yeh et
al., 1984, Journal of Chromatography 288:157-165.
Comparison of the macrocin O-methyltransferase activi-
ties in the transformed, GS28/pHJL280, and parental,
GS28, strains showed a 60-to-100-fold increase of enzyme

25   and 14-to-18-fold increase of tylosin production in the
transformed strain. Tylosin production was assayed and
determined in substantial accordance with the teaching
of Baltz and Seno, 1981, Antimicrobial Agents and
Chemotherapy 20:214-225; and Kennedy, J.H., 1983,

30   Journal of Chromatography 281:288-292.

Example 4

Construction of Streptomyces fradiae GS15/pHJL280

5   The desired strain was constructed in substan-
tial accordance with the teaching of Example 3 except
that Streptomyces fradiae GS15, rather than S. fradiae
GS28, was used. The desired strain was cultured for 72
hours and produced macrocin O-methyltransferase and
10   tylosin at levels above that produced in the un-
transformed S. fradiae GS15 strain, which produces no
readily detectable tylosin.

Example 5

15

Construction of Streptomyces fradiae GS15/pHJL284

       The desired strain was constructed in substan-
tial accordance with the teaching of Example 4 except
20   that plasmid pHJL284, rather than plasmid pHJL280, was
used. The desired strain was cultured and produced
macrocin O-methyltransferase and tylosin at levels above
that produced in the untransformed S. fradiae GS15
strain.

25

Example 6

Construction of Streptomyces fradiae GS16/pHJL280

5   The desired strain was constructed in substan-
tial accordance with the teaching of Example 3 except
that Streptomyces fradiae GS16, rather than S. fradiae
GS28, was used. The desired strain was cultured and
produced the tylE gene product, demethylmacrocin
10   O-methyltransferase, and tylosin at levels above that
produced in the untransformed strain. The
demethylmacrocin O-methyltransferase activity and
tylosin production respectively are assayed and deter-
mined in substantial accordance with the
15   above-referenced procedures, except that
demethylmacrocin is substituted for macrocin as
substrate.

Example 7

20

Construction of Streptomyces fradiae GS76/pHJL280

          The desired strain was constructed in substan-
tial accordance with the teaching of Example 3 except
25   that Streptomyces fradiae GS76, rather than S. fradiae
GS28, was used. The desired strain was cultured and
produced the tylD and tylH gene products and tylosin at
levels above that produced in the untransformed strain.

Example 8

Construction of Streptomyces fradiae GS48/pHJL280

5   The desired strain was constructed in substan-
tial accordance with the teaching of Example 3 except
that Streptomyces fradiae GS48, rather than S. fradiae
GS28, was used. The desired strain was cultured and
produced the tylD gene product and tylosin at levels
10   above that produced in the untransformed strain.

Example 9

Construction of Streptomyces fradiae GS52/pHJL284

15   The desired strain was constructed in substan-
tial accordance with the teaching of Example 3 except
that Streptomyces fradiae GS52 and plasmid pHJL284,
rather than S. fradiae GS28 and plasmid pHJL280, were
20   used. The desired strain was cultured and produced the
tylC gene product and tylosin at levels above that
produced in the untransformed strain.

Example 10

25

Specific Activity of Rate-Limiting Enzymes and
Increased Tylosin Production Using the Present Method

           The following Tables demonstrate the effec-
30   tiveness of the present method. All transformants
listed in the Tables were obtained in substantial
accordance with the procedure of Example 3. The results
indicated in Tables XII and XIII were obtained from
strains cultured in fermentation media (Baltz and Seno,

1981, Antimicrobial Agents and Chemotherapy 20:214-225)
that also contained 20 µg/ml thiostrepton if the strain
being cultured harbored a plasmid. Note that the
transformed strains listed in Tables XII and XIII are
5 low tylosin-producing, or produce amounts of tylosin
that are not readily detectable, and were cultured in
the presence of selective pressure (thiostrepton) for
plasmid maintenance as an autonomously replicating
vector.

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Table XII

Specific Activity of the tylF Gene Product, Macrocin O-methyltransferase (MOMT)

	Strain	Transforming Plasmid	MOMT Specific Activity			
			2 days[1]	3 days	4 days	6 days
Run 1	GS15	pHJL210[4]	0	0	0	0
	GS15	pHJL280	1.14	1.93	NT	NT
	C4[2]	None	Not tested (NT)	0.35	0.16	NT
Run 2	GS15	pHJL210	0	0	0	0
	GS15	pHJL280	4.2	3.2	2.2	1.8
	C4	None	0.8	1.0	0.9	0.9
	T1405[3]	None	0.9	1.2	1.5	1.4
Run 3	GS28	None	0	0.01	0.03	NT
	GS28	pHJL210	0	0	0	NT
	GS28	pHJL280	0.8	0.7	1.0	NT
	GS28	pHJL284	0.9	1.2	0.9	NT
	C4	None	0.2	0.6	0.5	NT

[1] days in fermentation.

[2] the strain from which GS15 and GS28 were derived.

[3] a strain derived from C4.

[4] the cloning vector into which the tyl genes were inserted to obtain plasmids pHJL280 and pHJL284.

Table XIII

Specific Activity of the tylE Gene Product, Demethylmacrocin
O-methyltransferase (DMOMT)

Strain	Transforming Plasmid	DMOMT Specific Activity			
		2 days*	3 days	4 days	
GS16	pHJL210	0	0	0	
GS16	pHJL280	1.8	3.7	4.0	
GS16	pHJL280	3.8	1.7	3.0	
GS16	pHJL284	1.3	1.6	2.2	
C4	pHJL210	0.7	1.3	1.5	
C4	pHJL210	0.2	1.1	1.9	
C4	None	0.4	1.5	1.0	

The results in Table XIV were obtained from
transformants of high tylosin-producing strains that
were cultured post-transformation to obtain integrants,
transformants in which all or part of the plasmid DNA

5   has integrated into the genome of the host cell. Two
methods were used to obtain the integrants. In the
first method, transformants are passaged onto selective
(contains thiostrepton) and nonselective plates and
incubated about 16 hours at 29°C to obtain single

10 colonies. The single colonies on the nonselective
plates that were thiostrepton-resistant on the selective
plate are repassaged several times in the same manner
until a single colony was found to be relatively stable
without selection. In the second method for obtaining

15 integrants, the transformants were nonselectively
passaged several times by transferring spores from the
surface of the plate using a cotton swab. After several
passages, the colonies are grown in non-selective,
liquid media (TSB), homogenized, fragmented by sonica-

20 tion, diluted, and plated on selective and nonselective
media to identify relatively stable integrants. Other
methods of obtaining integrants are apparent to those
skilled in the art, and the present method is not
limited to a particular method of obtaining integrants.

25   Relatively stable integrants were used to
inoculate vegetative medium (complex vegetative medium
contains, per liter, 10 g of corn steep liquor, 5 g of
yeast extract, 5 g of soybean grits, 3 g of calcium
carbonate, and 4.5 g of crude soybean oil, and the pH is

30 adjusted to 7.8 with NaOH. TSB is also a suitable

vegetative media) without thiostrepton (no selective
pressure), and the vegetative culture was used to
inoculate (10% inoculum) the fermentation medium, which
also lacked thiostrepton. Fermentations were run at

5   260 rpm at 29°C for seven days. The total macrolide
content of the fermentation broth was measured by
extraction with methanol:$CHCl_3$, reading the absorbance
at 290 nm, and comparing to a standard curve. Tylosin
factors were identified by spotting the fermentation

10 broth onto silica-gel-TLC plates and developing the
plates with a solvent system of 95:5
ethylacetate:diethylamine. The concentration of indi-
vidual macrolide components was the total A_{290} times the
percentage of each component as determined by HPLC.

15

Table XIV

strain	% Thiostrepton Resistant	Transforming Plasmid	DMOMT Specific Activity	MOMT Specific Activity	Tylosin*
C4	0	None	0.59	0.14	1
C4	9.5	pHJL280	Not tested (NT)	NT	1.10
C4	5.9	pHJL280	NT	NT	0.97
T1405	0	None	1.0	0.17	1.14
T1405	50	pHJL280	0.91	0.26	1.52
T1405	8.7	pHJL280	NT	NT	1.21
T1405	11	pHJL280	NT	NT	1.07
T1405	6.4	pHJL280	NT	NT	1.21
T1405	18	pHJL280	2.5	0.43	1.60
T1405	4.6	pHJL280	NT	NT	0.98
T1405	16	pHJL280	NT	NT	1.07
T1405	12	pHJL280	NT	NT	1.10
T1405	18	pHJL280	NT	NT	1.28
T1405	25	pHJL280	NT	NT	1.28
T1405	56	pHJL280	0.82	0.22	1.45

*Relative to C4 strain

Example 11

Preparation of Tylosin

A sporulated culture of Streptomyces fradiae containing the plasmids provided by the present invention can be produced by growing the organism on a nutrient agar slant having the following composition:

Yeast extract	1.0 g
Beef extract	1.0 g
Hydrolyzed casein ("N-Z-Amine-Type A," sold by the Sheffield Chemical Co.)	2 g
Dextrin	10 g
Cobaltous chloride heptahydrate	20 mg
Agar	20 g
Water	1 l

The pH of the medium is adjusted to pH 7.3 by the addition of sodium hydroxide.

The slant is inoculated with spores of the desired organism and is incubated for five days at about 30°C. The sporulated culture growth on the slant is covered with water, and the slant is scraped gently to remove the spores to provide an aqueous spore suspension.

1 ml. of the spore suspension is used to inoculate under aseptic conditions a 100 ml. portion of a sterile vegetative culture medium having the following composition:

	G.
Glucose	15
Soybean meal	15
Corn steep solids	15
Sodium chloride	5
Calcium carbonate	2
Tap water, added to make a total of volume of 1 l.	

The inoculated vegetative medium is incubated at about 30°C. for 48 hours, during which time the incubate is shaken at the rate of 114 cycles per minute on a reciprocal shaker having a 2-inch stroke.

5 ml. of the vegetative inoculum are used to inoculate aseptically 100 ml. portions of the following sterilized production medium contained in 500 ml. Erlenmeyer flasks:

Soybean meal	15 g
Casein	1 g
Crude glucose syrup	20 ml
Calcium carbonate	2.5 g
Sodium nitrate	3 g
Tap water, added to make a total of volume of 1 l.	

The inoculated culture then is incubated for
100 hours at about 26-28°C. During the incubation
period, the incubate is shaken at 114 revolutions per
minute on a reciprocal shaker having a 2-inch stroke.
5   The pH of the starting medium is about pH 6.5, and at
the end of the incubation period, the pH of the medium
generally increases to about pH 7.5.

The fermented culture broth is filtered to
remove the mycelium and other undissolved solids. The
10   filtered broth contains the tylosin.

Example 12

Alternate Preparation of Tylosin

15

A sporulated culture of the desired trans-
formed microorganism is produced by growing the organism
on a nutrient agar slant having the following
composition.

20

Tomato paste-oatmeal Agar:	G
Tomato paste	20
Pre-cooked oatmeal	20
Agar	15
Water, added to make a total volume of 1 l.	

25

The slant is inoculated with spores of the
organism and the inoculated slant is incubated for 9

days at a temperature of about 30°C. After incubation,
the sporulated culture on the slant is covered with
water, and the surface of the slant is scraped gently to
remove the spores to obtain an aqueous spore suspension.

5   Employing aseptic techniques, one-half of the
inoculum obtained from one agar slant is used for
inoculating a 500 ml. portion of a sterilized vegetative
culture medium having the following composition con-
tained in a 2 l. Erlenmeyer flask:

10

Corn-steep yeast I: G.
     Glucose   .   15
     Corn steep solids   5
     Yeast   5
15   Calcium carbonate   5
     Water, added to make a total volume of 1 l.

The incubation is carried on at 28°C for 48
hours with shaking at 110 cycles per minute on a recip-
20   rocal shaker having a 2-inch stroke.

          0.25 gal. of the vegetative inoculum from the
flask is added aseptically as an inoculum to 250 gal. of
the sterile corn steep yeast I medium described above
contained in an iron 350 gal fermentor. 0.025 gal. of
25   Antifoam A (an antifoam product sold by The Dow Corning
Company) is added to the culture medium to prevent
excessive foaming, and additional quantities are added
as needed during the fermentation. The inoculated
medium is fermented for 24 hours at a temperature of
30   28°C. During fermentation, the medium is aerated with
sterile air at a rate of 27 cubic feet per minute and is

agitated with two 16-inch impellers operated at 160
revolutions per minute.

To a 1700 gal. iron fermentor are added 1200
gal. of a medium having the following composition.

Corn steep soy XII:	Kg.
Glucose	30
Soybean oil meal	15
Corn steep solids	5
Crude soybean oil	10
Calcium carbonate	2
Sodium chloride	5
Water, added to make a total volume of 1000 l.	

The medium is inoculated with 96 gal. of the
inoculum grown in the fermentation tank. The fermenta-
tion is carried on at 28°C for four days, and any
foaming is controlled by the addition as needed of
"Larex" No. 1 (an antifoam product sold by Swift &
Company). The fermentation medium is aerated by the
addition of sterile air at the rate of 128 cubic feet
per minute and is agitated with two 24-inch impellers
operated at 130 revolutions per minute.

600 pounds of "Silflo" (a diatomaceous earth
filter aid sold by The Silfo Company) are added to the
broth, and the mixture is filtered. The filtrate is
adjusted to pH 8.5 by the addition of 20 percent sodium
hydroxide, 500 gal. of chloroform are added, the mixture
is stirred for 30 minutes, and the chloroform layer
which is in the form of an emulsion is decanted. The
chloroform extraction is repeated twice with 500-gal.
portions of chloroform. The chloroform emulsions which

contain the tylosin are combined and are passed through
a De Laval separator to break the emulsion, and the
chloroform solution is then concentrated in vacuo to a
volume of 25 l. The impurities are largely removed from

5   the solution by passing it over a column 6 inches in
diameter containing 10 kg. of activated carbon such as
that sold by the Pittsburgh Coke and Chemical Co. The
carbon column is washed with 16 l. of chloroform, and
the combined chloroform effluents containing the tylosin

10 are concentrated in vacuo to a volume of about 2 l. The
chloroform concentrate is added slowly with stirring to
20 l. of petroleum ether, the mixture is stirred for 15
minutes, it is filtered to remove the white, amorphous
precipitate of tylosin.

15   The amorphous tylosin is crystallized by
dissolving it in 355 ml. of acetone, filtering the
acetone mixture to remove a slight haze, and slowly
adding the filtered acetone mixture with gentle stirring
to 20 l. of water at 5°C. The aqueous, acetone solution

20 of tylosin is permitted to stand at room temperature
with gentle stirring to permit the acetone to evaporate
slowly, whereupon tylosin crystallizes out. The tylosin
crystals are removed by filtration and are dried in
vacuo at room temperature. Tylosin has a melting point

25 of about 127-132°C.

Example 13

Preparation of Tylosin Tartrate

5   5 g. of crystalline tylosin are dissolved in
100 ml. of acetone, and 1.5 g. of D-tartaric acid
dissolved in 20 ml. of acetone are added with stirring.
The solution is permitted to stand at room temperature
whereupon the tartrate salt of tylosin crystallizes out
10   of the solution. The crystals of the tartrate salt of
tylosin are removed by filtration, are washed with
acetone, and are air-dried. The crystalline tartrate
salt of tylosin melts at about 140-146°C.

15   Example 14

Preparation of Tylosin Gluconate

           1.03 g. glucono-delta lactone are dissolved in
20   10 ml. of water, and the aqueous solution is warmed to
85°C. for two hours to cause hydrolysis of the lactone
to gluconic acid. 15 ml. of warm methanol are added to
the aqueous solution. 5 g. of tylosin dissolved in 10
ml. of methanol are added to the methanol mixture with
25   stirring. The tylosin methanol mixture is permitted to
stand overnight at room temperature. The methanol is
removed from the mixture by evaporation in vacuo at room
temperature. After the methanol is removed, 40 ml. of
water are added to the aqueous tylosin mixture. The
30   diluted mixture is filtered, and the filtrate containing
the tylosin is freeze-dried, producing a white solid
consisting of the gluconate salt of tylosin. Tylosin
gluconate salt melts at about 114-117°C.

Example 15

Preparation of Tylosin Hydrochloride

5   890 mg. of tylosin are dissolved in 200 ml. of
ether. The ether mixture is acidified by the addition
of 0.082 ml. of 12 N hydrochloric acid. The precipitate
of the hydrochloride salt of tylosin which forms is
filtered off, is washed with ether, and is dried in
10   vacuo. The hydrochloride salt of tylosin is
recrystallized from an ethanol-ether mixture. The
hydrochloride salt of tylosin has melting point of about
141-145°C.

Claims

1.   A method for increasing the antibiotic- or antibiotic precursor-producing ability of an antibiotic-producing microorganism, which comprises culturing a microorganism which produces an antibiotic or antibiotic precursor by a biosynthetic pathway, said microorganism being transformed with a DNA cloning vector or portion thereof which contains an antibiotic or antibiotic-precursor biosynethetic gene coding for expression of a rate-limiting enzyme or gene product of the biosynthetic pathway, under conditions suitable for cell growth, expression of the antibiotic or antibiotic-precursor biosynthetic gene and production of the antibiotic or antibiotic precursor, provided that the culturing process provides an increase in the antibiotic-producing ability of the microorganism.

2.   A method as claimed in Claim 1 in which the microorganism is Streptomyces, Cephalosporium, or Penicillium.

3.   A method as claimed in Claim 1 or 2 in which the microorganism, antibiotic, antibiotic precursor, antibiotic biosynthetic pathway, and antibiotic biosynthetic gene respectively are Streptomyces, tylosin, a tylosin precursor, tylosin biosynthetic pathway, and a tylosin biosynthetic gene.

4.   A method as claimed in Claim 3 in which the microorganism is S. fradiae, S. rimosus, and S. hygroscopicus.

5.   A method as claimed in Claim 4 in which
the tylosin biosynthetic gene is tylC, tylD, tylE,
tylF, tylH, tylJ, tylK, tylL, or tylM.

6.   A method as claimed in any one of Claims
1 to 5 in which the biosynthetic gene is tylF.

7.   A method as claimed in Claim 1 in which
the cloning vector is plasmid pHJL280, pHJL284, pHJL309,
pHJL311, or pHJL315.

8.   A method as claimed in claim 1 in which
the microorganism transformed with a cloning vector is
Streptomyces fradiae GS15/pHJL280, S. fradiae
GS15/pHJL284, S. fradiae GS15/pHJL311, S. fradiae
GS15/pHJL315, S. fradiae GS28/pHJL280, S. fradiae
GS28/pHJL284, S. fradiae GS28/pHJL311, S. fradiae
GS28/pHJL315, S. fradiae GS16/pHJL280, S. fradiae
GS16/pHJL315, S. fradiae GS48/pHJL280, S. fradiae
GS48/pHJL315, S. fradiae GS52/pHJL284, S. fradiae
GS52/pHJL311, S. fradiae GS76/pHJL280, or S. fradiae
GS76/pHJL315.

9.   A recombinant DNA cloning vector as
defined in claim 1.

10.   Plasmids pHJL280, pHJL284, pHJL309,
pHJL311, or pHJL315.

11.   A microorganism that is transformed with
a cloning vector as claimed in claim 9.

12.   A microorganism which is transformed with
a plasmid of Claim 10.

13.   A microorganism of Claim 12 which is E.
coli K12 HB101/pHJL280, E. coli K12 HB101/pHJL284, E.
coli K12 HB101/pHJL309, E. coli K12 HB101/pHJL311, or E.
coli K12 JM109/pHJL315.

14. A microorganism as claimed in Claim 12 which is Streptomyces.

15. A microorganism as claimed in Claim 14 which is Streptomyces fradiae.

16. S. fradiae GS15/pHJL280, S. fradiae GS15/pHJL284, S. fradiae GS15/pHJL311, S. fradiae GS15/pHJL315, S. fradiae GS28/pHJL280, S. fradiae GS28/pHJL284, S. fradiae GS28/pHJL311, S. fradiae GS28/pHJL315, S. fradiae GS16/pHJL315, S. fradiae GS48/pHJL280, S. fradiae GS48/pHJL315, S. fradiae GS52/pHJL284, S. fradiae GS52/pHJL311, S. fradiae GS76/pHJL280, or S. fradiae GS76/pHJL315.

17. A DNA sequence that encodes a gene for the tylC, tylD, tylE, tylF, tylH, tylJ, tylK, tylL, or tylM biosynthetic genes.

18. A DNA sequence which encodes the promoter and translational-activating sequence of the tylC, tylD, tylE, tylF, tylH, tylJ, tylK, tylL, or tylM biosynthetic genes.

19. A DNA sequence which encodes the promoter and translational-activating sequence of the tylF gene.

20. A DNA sequence as claimed in Claim 19 encoding the tylF promoter that is

5'-CTG TGT CAG GTC GCC CGT GGT GAC GGG CTC CGG GGC GGC GGC GCG
|
R

GGC GGC CGA CCT TGA CAT ACC CGC GGC CGG GCT CCT CGT TCC GGC GCG
GCC CGC GCC GAT AGC GTC CGT CCT CAC CGG CTC CGG CGT CCG CGT CCC
CGC CGG GAC GTG CCA CCT CTC CCG ACC CCG CGA GCC GAT CGA CCC GCT
ACT GGA GGA CCC-3'

wherein A is a deoxyadenyl residue; G is a deoxyguanyl
residue; C is a deoxycytidyl residue; and T is a
thymidyl residue and wherein R is a sequence of deoxyri-
bonucleotide that is complementary to said DNA sequence
5   depicted such that A is paired with T; T is paired with
A; G is paired with C; and C is paired with G.

        21. A process for preparing an antibiotic, an
antibiotic precursor, or a pharmaceutically acceptable
salt thereof, which comprises culturing a microorganism
10 which produces an antibiotic or antibiotic precursor
through an antibiotic biosynthetic pathway, said micro-
organism being transformed with a DNA cloning vector, or
portion thereof, in a culture medium containing assimi-
lable sources of carbon, nitrogen and inorganic salts
15 under aerobic fermentation conditions characterized in
that the DNA cloning vector, or portion thereof, com-
prises an antibiotic biosynthetic gene which codes for
the expression of a rate-limiting enzyme or gene product
of the antibiotic biosynthetic pathway, said antibiotic
20 biosynthetic gene being expressed under fermentation
conditions providing for an increase in the
antibiotic-producing ability of the microorganism.

        22. A process as claimed in Claim 21 in which
the antibiotic or antibiotic-precursor is tylosin or a
25 tylosin-precursor.

5

23.   A DNA sequence encoding the _tylF_ gene.

24.   The _tylF_ gene.

25.   The gene claimed in claim 24 having the sequence:

```
                 10          20          30          40
5'-TTC GCG GGA TGG ATG CTG ACC CGG GGG TCG GCC AGC AGC GCC CGG ACG


       50          60          70          80          90
   TGA TCT GGC GGG AGA TCA GCC AGA CCG GCG CCC CGT CCC ACA GCT CGG


          100         110         120         130         140
   CCC GGG CGA TCG GCT CCT CCG CCC GGA GGG CGG CGT ACT GCT CGG GAG


          150         160         170         180         190
   GGC TGA AGG GAC AGG TGC GGG CGA CCG GCC AGG CGA TGC TGC GCC GGC


          200         210         220         230         240
   CTG CGG CCC CGT CGG TGT CGT TGG CGC GTG CTG CGG GCA ACA GAA TCC


          250         260         270         280
   CCT TTT GTG ACG GGC GGG CGT CCC CGG ACG AGG ACA CGA CTC GCT GCG


      290         300         310         320         330
   GCC TCA ACG AAA ACA CCG TGT CCG GTG CCC AGG CCA CGA ACG GTG ACC


      340         350         360         370         380
   GGT CTG TGT CAG GTC GCC CGT GGT GAC GGG CTC CGG GGC GGC GGC GCG
```

```
        390           400           410           420           430
        GGC GGC CGA CCT TGA CAT ACC CGC GGC CGG GCT CCT CGT TCC GGC GCG


5                     440           450           460           470           480
        GCC CGC GCC GAT AGC GTC CGT CCT CAC CGG CTC CGG CGT CCG CGT CCC


                      490           500           510           520
        CGC CGG GAC GTG CCA CCT CTC CCG ACC CCG CGA GCC GAT CGA CCC GCT

10
        530           540           550           560           570
        ACT GGA GGA CCC GTG GCA CCT TCC CCG GAC CAC GCC CGC GAT CTC TAC
                      VAL ALA PRO SER PRO ASP HIS ALA ARG ASP LEU TYR
                                          5                       10

15
        580           590           600           610           620
        ATC GAG CTG CTG AAG AAG GTC GTC TCG AAC GTC ATC TAC GAG GAC CCC
        ILE GLU LEU LEU LYS LYS VAL VAL SER ASN VAL ILE TYR GLU ASP PRO
                      15                  20                  25

20
        630           640           650           660           670
        ACC CAT GTG GCG GGG ATG ATC ACC GAC GCG TCG TTC GAC CGG ACG TCC
        THR HIS VAL ALA GLY MET ILE THR ASP ALA SER PHE ASP ARG THR SER
                      30                  35                  40

25
        680           690           700           710           720
        CGT GAG AGC GGC GAG GAC TAC CCC ACG GTC GCC CAC ACG ATG ATC GGC
        ARG GLU SER GLY GLU ASP TYR PRO THR VAL ALA HIS THR MET ILE GLY
        45                  50                  55                  60
```

```
              730           740           750           760
       CTC AAG CGT CTG GAC AAT CTC CAC CGG TGC CTC GCG GAC GTC GTG GAG
       LEU LYS ARG LEU ASP ASN LEU HIS ARG CYS LEU ALA ASP VAL VAL GLU
 5                      65              70              75


              770           780           790           800           810
       GAC GGC GTC CCC GGT GAC TTC ATC GAG ACC GGG GTG TGC CGC GCG CCG
       ASP GLY VAL PRO GLY ASP PHE ILE GLU THR GLY VAL CYS ARG ALA PRO
 10                     80              85              90


              820           830           840           850           860
       TGC ATC TTC GCC CGC GGA CTG CTG AAC GCG TAC GGC CAG GCC GAC CGC
       CYS ILE PHE ALA ARG GLY LEU LEU ASN ALA TYR GLY GLN ALA ASP ARG
 15                     95             100             105


              870           880           890           900           910
       ACC GTC TGG GTC GCC GAC TCC TTC CAG GGC TTT CCC GAG CTG ACC GGG
       THR VAL TRP VAL ALA ASP SER PHE GLN GLY PHE PRO GLU LEU THR GLY
 20           110             115             120


              920           930           940           950           960
       TCC GAC CAC CCG CTG GAC GTC GAG ATC GAC CTC CAC CAG TAC AAC GAG
       SER ASP HIS PRO LEU ASP VAL GLU ILE ASP LEU HIS GLN TYR ASN GLU
 25    125             130             135             140


              970           980           990           1000
       GCC GTG GAC CTG CCC ACC AGC GAG GAG ACC GTG CGG GAG AAC TTC GCC
       ALA VAL ASP LEU PRO THR SER GLU GLU THR VAL ARG GLU ASN PHE ALA
 30                     145             150             155
```

```
         1010          1020          1030          1040          1050
     CGG TAC GGG CTG CTC GAC GAC AAC GTC CGT TTC CTG GCG GGG TGG TTC
     ARG TYR GLY LEU LEU ASP ASP ASN VAL ARG PHE LEU ALA GLY TRP PHE
  5               160               165               170


         1060          1070          1080          1090          1100
     AAG GAC ACC ATG CCG GCT GCG CCC GTG AAG CAG CTC GCG GTG ATG CGC
     LYS ASP THR MET PRO ALA ALA PRO VAL LYS GLN LEU ALA VAL MET ARG
 10               175               180               185


         1110          1120          1130          1140          1150
     CTG GAC GGC GAC TCC TAC GGC GCC ACC ATG GAT GTG CTC GAC AGC CTG
     LEU ASP GLY ASP SER TYR GLY ALA THR MET ASP VAL LEU ASP SER LEU
                  190               195               200


         1160          1170          1180          1190          1200
     TAC GAG CGG CTG TCG CCG GGC GGT TAC GTC ATC GTC GAC GAC TAC TGC
     TYR GLU ARG LEU SER PRO GLY GLY TYR VAL ILE VAL ASP ASP TYR CYS
 20  205               210               215               220


         1210          1220          1230          1240
     ATC CCG GCC TGC CGC GAG CGG TGC ACG ACT TCC GCG ACC GGC TCG GCA
     ILE PRO ALA CYS ARG GLU ARG CYS THR THR SER ALA THR GLY SER ALA
  2               225               230               235


     1250          1260          1270          1280          1290
     TCC GCG ACA CGA TCC ACC GGA TCG ACC GCC AGG GCG CTA TTG GCG GCA
     SER ALA THR ARG SER THR GLY SER THR ALA ARG ALA LEU LEU ALA ALA
 30               240               245               250
```

```
        1300           1310           1320           1330           1340
CAG CGG CTG AGT CGT TCC GCC CGA GAG CCC GAC GAG AGC AGG AGA TAT
GLN ARG LEU SER ARG SER ALA ARG GLU PRO ASP GLU SER ARG ARG TYR
        255                          260                 265


        1350           1360           1370           1380           1390
GCG AGA CAC GAC GCG CCC GCT CGG CAT TGA GGG AGC GTG GGT GAT CCA
ALA ARG HIS ASP ALA PRO ALA ARG HIS
        270                   275


        1400           1410           1420           1430           1440
GCC GGA GAT CCA TCC GGA CCG GCG CGG CGA GTT CCA CGC GTG GTT CCA


              1450           1460           1470           1480
GAG CCA GCC GAG TTC CGG CGG CTG ACC GGT CAC TCC TTC TCC GTG CCG


    1490           1500           1510           1520           1530
CAG GTC GTC AAT ATC GCG TGT CCC GGA AAG GCG CCG CTG CGG CAT CCA


        1540           1550           1560           1570           1580
CTT CTG CCG AGG TGC CAC CGG GCC GAG GCC AAG TAC AGC GGC GTG TGT


        1590           1600           1610           1620           1630
GCA GGG CGC CGG TGT CGA GGT CGT CGT CGA CGC GCC GGT GTC GAG GTC


        1640
GTC GTC GAC-3'
```

wherein A is deoxyadenyl residue; G is a deoxyguanyl residue; C
is a deoxycytidyl residue; and T is a deoxythymidyl residue.

0238323

26. The amino acid sequence encoded by nucleotides 541 to 1371 of the tylF gene defined in claim 25.

5

FIG. I
The Tylosin Biosynthetic Pathway
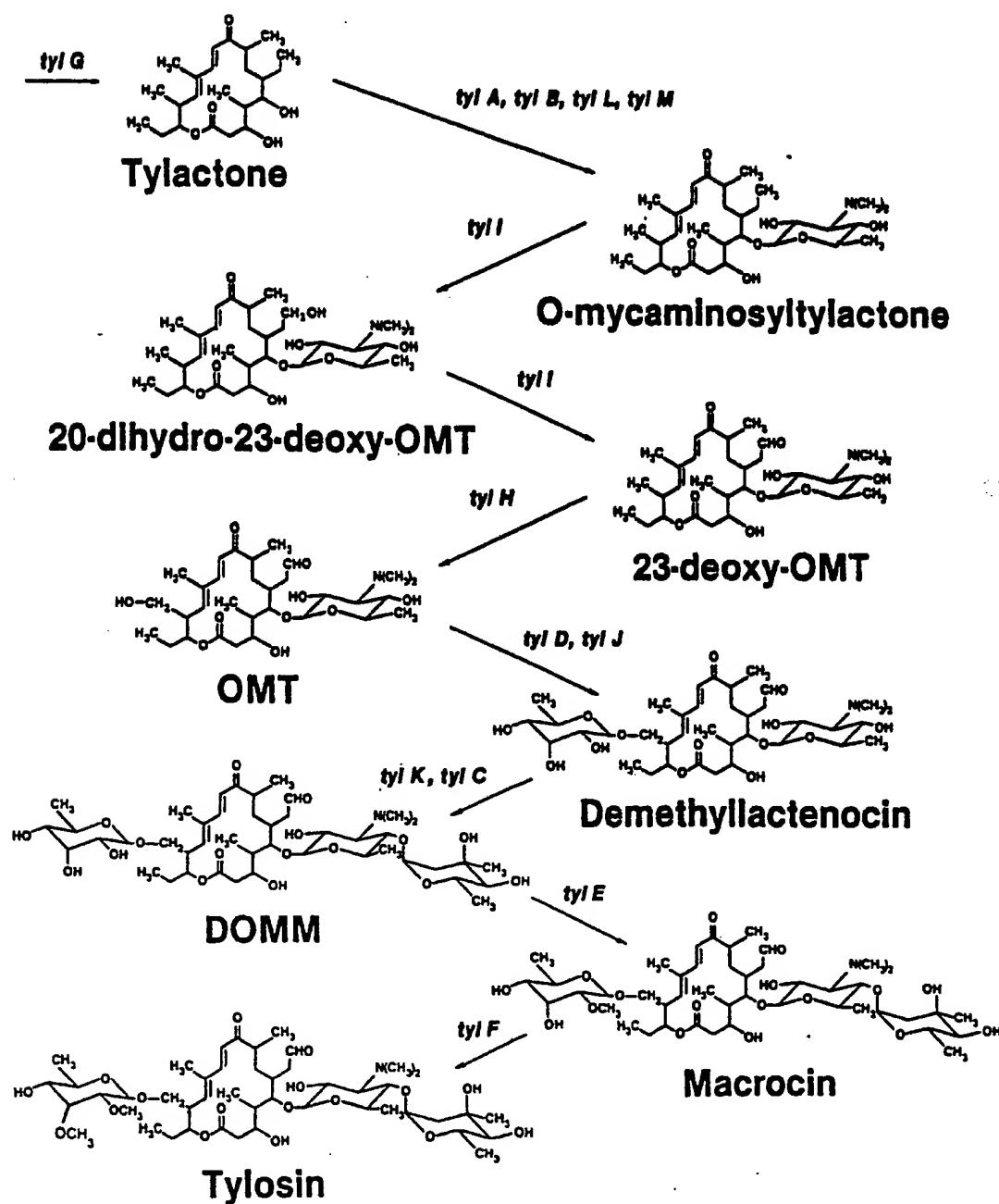
FIG.2

Restriction Site and Function Map of Plasmid pHJL280 (26.94 kb)
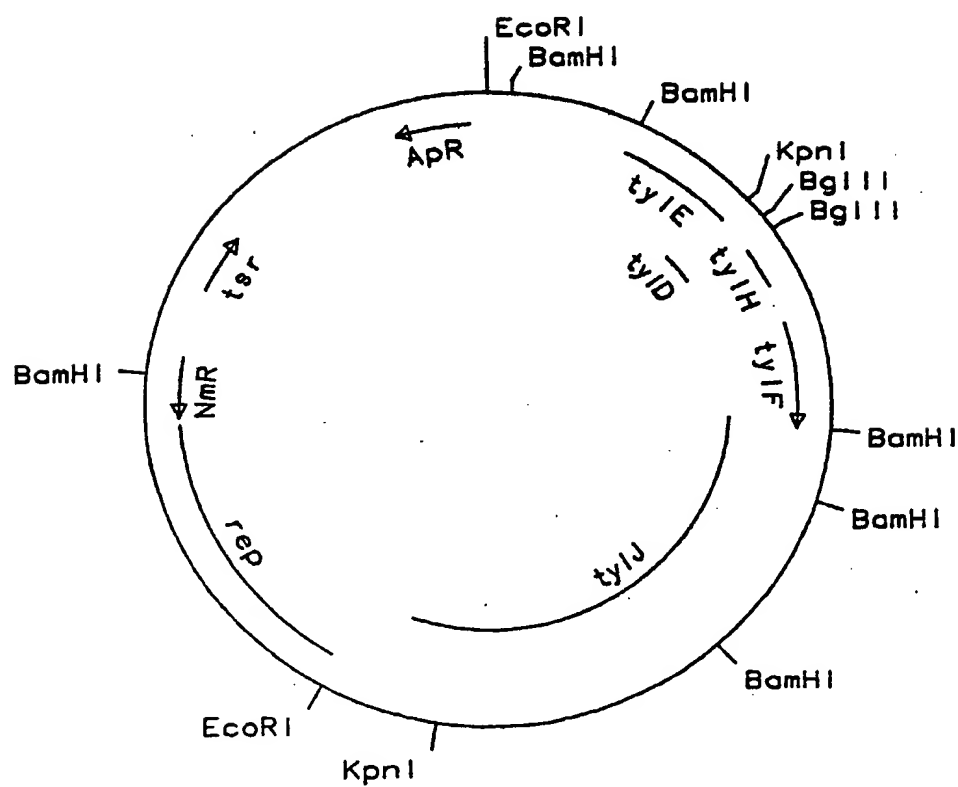
FIG.3

Restriction Site and Function Map of Plasmid pHJL284
(26.94 kb)
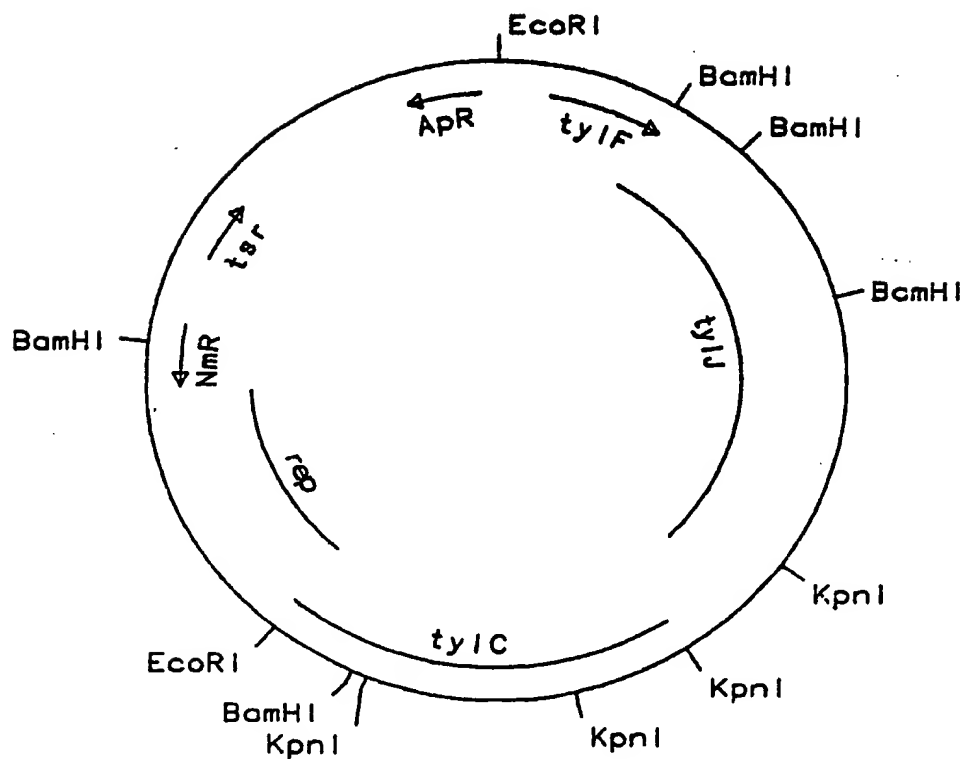
FIG.4

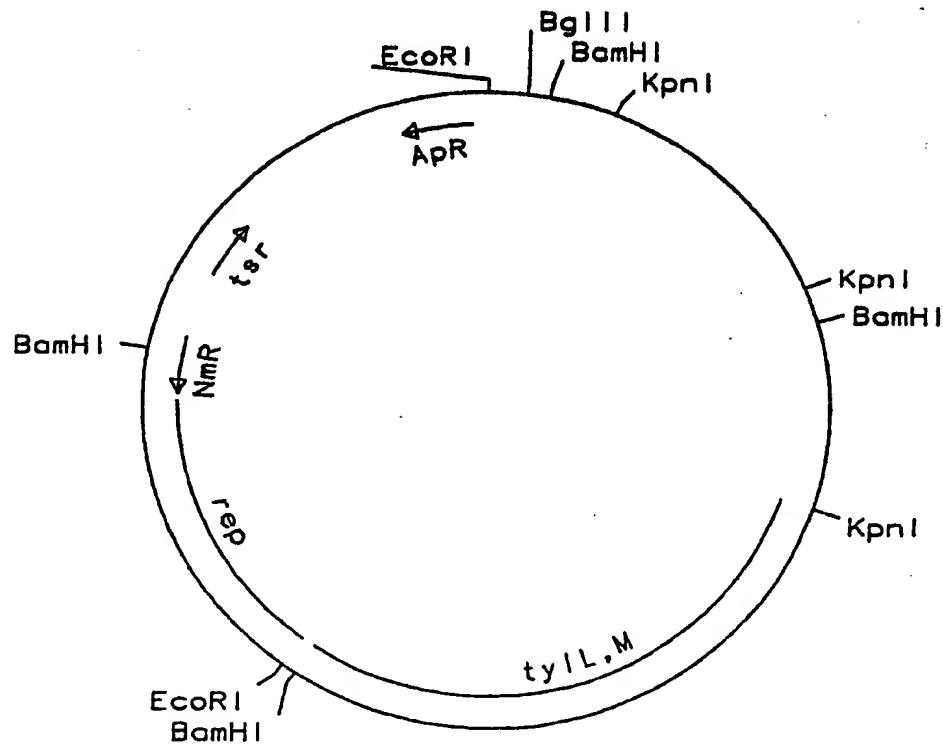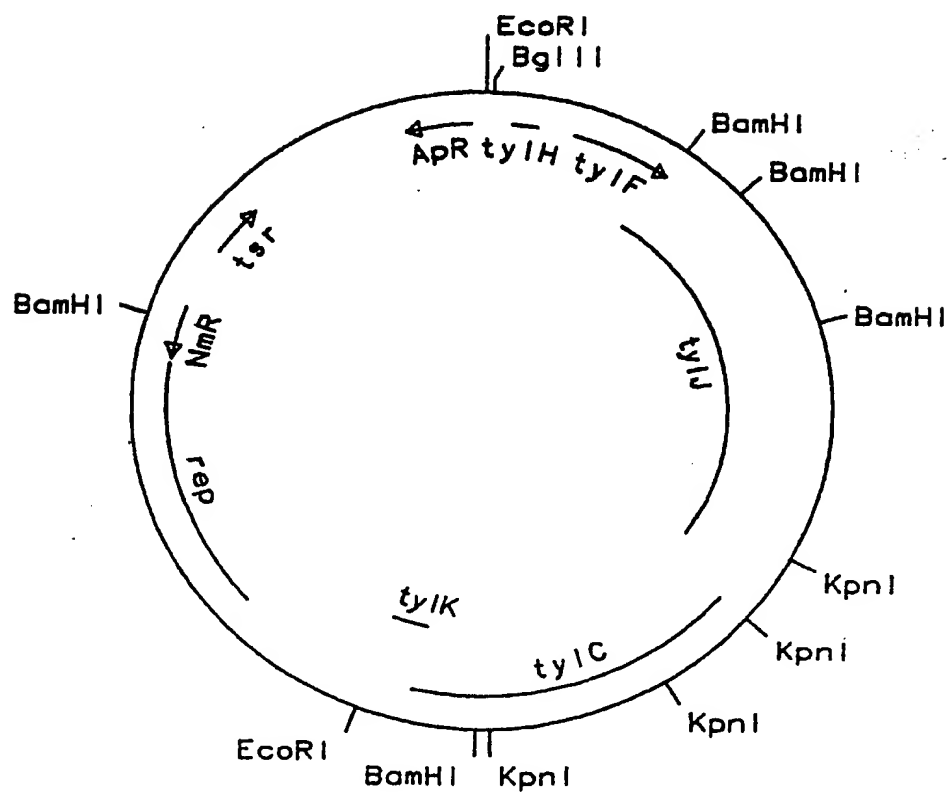## Restriction Site and Function Map of Plasmid pHJL309 (28.24 kb)

FIG.5

Restriction Site and Function Map of Plasmid pHJL311
(30.84 kb)

FIG.6

Restriction Site and Function Map of
Plasmid pHJL315
(38.83 kb)

FIG.7
Chromosomal Organization of the Tylosin Biosynthetic Genes